Research Article

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Stacked traits conferring multiple resistance to imazamox and glufosinate in soft wheat

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Abstract

BACKGROUND: Conventional crossing of soft wheat cultivars resistant to imazamox and glufosinate resulted in two (Rados and Helter) lines resistant to both herbicides. Stacked traits conferring this dual herbicide resistance in these lines, compared with a susceptible (S) cultivar, were characterized.

RESULTS: Rados and Helter lines were ~ 18-fold more resistant (R) to glufosinate, and between 15.1 and 19.8-fold more resistant to imazamox than the S cultivar. Resistance to glufosinate and imazamox decreased up to 12% and 50%, respectively, when the herbicides were applied sequentially. The basal activities of the acetolactate and glutamine synthases were similar between R and S plants. Rados and Helter lines were 11.7- and 17.7-fold more resistant to imazamox than the S cultivar, due to the Ser653–Asn mutation in their *imi*-ALS genes. R lines, susceptible to glufosinate at the target site level, showed lower ammonia accumulation evidencing the activity of the phosphinothricin acetyl transferase. Absorption and translocation patterns for ^{14}C-imazamox and ^{14}C-glufosinate were similar between R and S cultivars and so do not contribute to resistance.

CONCLUSION: Stacked traits conferring dual herbicide resistance to the lines Rados and Helter come from the resistant parents. These R lines are potential tools for weed management in wheat production, mainly via herbicide rotation.

Supporting information may be found in the online version of this article.

Keywords: acetolactate synthase; glutamine synthase; herbicide resistance crops; *N*-acetyl-glufosinate; phosphinothricin acetyl transferase; *Triticum aestivum*

1 INTRODUCTION

Wheat (*Triticum* spp.) is a crop of great economic importance with current production estimated at > 720 million tons per year worldwide,[1] used for the production of human and animal food. Weeds are major biotic factors in crop production, competing for soil, water, light and nutrients.[2] In wheat, weeds can result in a reduction in yield of up to 50%.[3]

Plant transformation methods (biolistic, electroporation, microinjection, *Agrobacterium tumefaciens*-mediated cell transformation, etc.), conventional plant breeding (plant selection, outcrossing with elite cultivars, mutagenesis, etc.), and combinations thereof have delivered herbicide-resistant (HR) crops that offer significant advantages for weed control.[4,5] These crops were introduced in the mid-1990s and adopted quickly by farmers. However, the use of HR crops resistant to a single mode of action and their improper use has led to the rapid expansion of a wide range of HR weeds, decreasing the value of this technology.[6] Judicious adoption of HR crops and their associated agronomic practices may help to maintain the biodiversity of agricultural lands and reduce the risk of weeds evolving herbicide resistance.[7] To improve this technology, HR crops with multiple or stacked traits are being developed.[8,9] Meanwhile, efforts are being made to implement new weed management strategies.[10]

Imazamox and ammonium glufosinate (glufosinate) are two of the most effective post-emergence herbicides. Imazamox is a systemic broad-spectrum herbicide that inhibits the enzyme acetolactate synthase (ALS), disrupting the synthesis of valine, leucine and isoleucine.[11] Glufosinate is a contact and non-selective herbicide that is minimally translocated, requiring complete coverage to ensure weed control.[12] This herbicide inhibits the activity of glutamine synthase (GS; EC 6.3.1.2), causing rapid accumulation of

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ammonia within the plant and damaging chloroplast and photosynthesis structures.[13]

Crop varieties resistant to imidazolinone herbicides (ALS inhibitors group) are based on non-transgenic Clearfield® technology developed by BASF. Its resistance is attributed to the Ser653–Asn mutation in ALS homologous genes located in chromosomes 6B and 6D of wheat.[14–16] Glufosinate resistance in crops is due to the *bar* (bialaphos resistance) or *pat* (phosphinothricin *N*-acetyltransferase) genes, isolated from *Streptomyces viridochromogenes* and *S. hygroscopicus,* respectively. These genes are 87% identical at the nucleotide sequence level, and both encode phosphinothricin acetyl transferase (PAT; EC 2.3.1.183),[13] responsible for deactivating the glufosinate to *N*-acetyl-glufosinate.[17] However, other resistance mechanisms should not be ruled out, such as foliar morphological alterations related to herbicide retention, decreased absorption and translocation, and detoxification of the herbicide by the plant.[11]

Crossing soft wheat (*Triticum aestivum* L.) cultivars resistant to imazamox and glufosinate using conventional methods resulted in lines resistant to both herbicides. The objectives of this study were: (i) to evaluate the degree of resistance to imazamox and glufosinate in two new resistant soft wheat varieties compared with a susceptible wheat cultivar, and (ii) to determine if resistance was accounted for entirely by the predicted stacked traits.

2 MATERIAL AND METHODS

2.1 Plant material, experimental conditions and herbicide treatments

The HR wheat lines Rados and Helter, each with multiple resistance to imazamox and glufosinate (IMA + GLU), came from crossing the glufosinate-resistant T-590 line (IAS/CSIC-Cordoba, Spain) with the Clearfield® Pantera cultivar resistant to imazamox (INIA-Carillanca, Chile). The Pantera cultivar has the Ser653–Asn mutation in the ALS-*imi1* and ALS-*imi2* genes.[15] The T-590 line contains the *bar* gene from pACH25 plasmid, which encodes the PAT enzyme.[18]

Seeds from the T-590 × Pantera cross (F_1 progeny) were sown in 15-cm Petri dishes containing two layers of filter paper moistened with 10 mL of distilled water and sealed with Parafilm. Seeds were maintained until germination in a growth chamber at 26/16 °C (day/night), a 14/10 h photoperiod, a light density of 850 mmol m^{-2} s^{-1}, and 60% relative humidity (RH). Seedlings were transplanted into 250-mL pots (one plant per pot) containing a mixture of peat and sand (1 : 1), and kept in a growth chamber under the same conditions.

Fifty wheat plants with three to four true leaves were treated with 40 g ai ha^{-1} of imazamox (Pulsar® 40, 4% w/v, BASF Española S. L., Tarragona, Spain) plus methyl oleate/methyl palmitate adjuvant (Dash HC®, 34.8% w/v, BASF Española S. L.) at 1.25 L ha^{-1}. Six hours after imazamox treatment, plants were treated again with 750 g ai ha^{-1} of glufosinate (Finale®, 15% w/v, Bayer Hispania S.L., Vila Seca, Spain). Herbicides were applied using a treatment chamber (Devries Manufacturing, Hollandale, MN, USA) equipped with a TeeJet 8002EVS flat fan nozzle calibrated at 250 kPa to deliver 250 L ha^{-1} of application volume at a height of 50 cm.

F1 individuals surviving herbicide application 21 days after treatment (DAT) were selected for self-pollination to obtain non-segregating lines. Two lines (Rados and Helter) with the best traits (height, spikes, grains, tillering, etc.) were multiplied by self-pollinating for five generations.[19] Twenty plants of each line were sown in 3-L pots (four plants per pot with five replicates) and treated with IMA + GLU (40 + 750 g ai ha^{-1}) as described above. The Spanish cultivar Gazul, a high-quality commercial variety susceptible (S) to both herbicides, was included as a control. No segregation was observed after herbicide application, and their herbicide resistance mechanisms were characterized.

2.2 Dose–response to imazamox and glufosinate

Susceptibility to imazamox and glufosinate in the Rados and Helter wheat lines was tested separately. The cultivar Gazul (S), as well as the resistant parents, the cultivar Pantera (parental resistant to imazamox) and the line T-590 (parental resistant to glufosinate) were included in the experiments. Wheat plants (one plant per pot in 250-mL pots) with three to four true leaves were treated with the following doses of imazamox: 0, 20, 40, 60, 80, 120, 160, 200 and 240 g ai ha^{-1} for the R plants and 0, 2.5, 5, 10, 20, 40 and 60 g ai ha^{-1} for the S plants; and glufosinate: 0, 300, 450, 600, 750, 900, 1200 and 1500 g ai ha^{-1} for the R plants and 0, 8.3, 16.7, 33.5, 75, 150 and 300 g ai ha^{-1} for the S plants. Methyl oleate/methyl palmitate adjuvant was added at 1.25 L ha^{-1} in all imazamox treatments. Parents resistant to a certain herbicide were considered susceptible to the opposite herbicide, i.e. the Pantera cultivar and T-590 line were treated with the glufosinate and imazamox doses, respectively, established for S plants. Herbicide applications were conducted as described above. At 21 DAT, plants were cut at ground level and weighed to determine the fresh weight. The experiments were conducted in a completely randomized design with 12 replicates per dose.

2.3 Interaction between herbicides

Three new dose–response assays were carried out on the Rados and Helter lines for each herbicide. The imazamox and glufosinate doses established for R plants above were applied again. However, when the wheat plants were treated with all doses of a particular herbicide, they received three additional separate doses of the opposite herbicide and vice versa. The additional doses were 20, 40 and 60 g ai ha^{-1} of imazamox, and 450, 600 and 750 g ai ha^{-1} of glufosinate. These rates correspond to the minimum, intermediate and maximum recommended field rates for each herbicide. Each additional dose of herbicides represented a new dose–response curve. The media and conditions were the same as in the previous dose–response assays. In all cases, glufosinate was applied in sequential application 6 h after imazamox. At 21 DAT, plants were cut at ground level and weighed to determine the fresh weight. The experiments were conducted in a completely randomized design with 12 replications per dose.

2.4 Foliar herbicide retention

Wheat plants with three to four true leaves were treated with a solution containing 40 + 750 g ai ha^{-1} of IMA + GLU, respectively, plus 1.25 L ha^{-1} of methyl oleate/methyl palmitate adjuvant plus 100 mg L^{-1} Na-fluorescein[20] under the same conditions as in the dose–response assays. Na-fluorescein was used as a labeling reagent to determine the amount of herbicide solution retained. After treatment, when the herbicide solution on the leaves had dried (20–25 min) at room temperature, plants were cut at ground level and shaken vigorously for 30 s in an Erlenmeyer flask containing 50 mL of 5 mM NaOH. The washing solution was recovered in glass flasks and the fluorescein absorbance was measured immediately at $490_{exc}/510_{em}$ nm (Hitachi F-2500 spectrofluorimeter). The tissues were stored in paper envelopes and dried in an oven at 80 °C for 72 h. Seven plants of each line or cultivar were used in a completely randomized design. Retention was expressed as µL of herbicide solution (IMA + GLU) per g of dry matter.

2.5 Enzyme activity of ALS and GS assays

Six grams (separated into two 3-g aliquots) of tissue from the youngest expanded leaves per wheat line were taken and immediately powdered using liquid N_2.

ALS enzyme activity was determined with 3 g (first aliquot) per wheat line following the methodology of Hatami *et al.*[21] Technical grade imazamox (2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-5-methoxymethylnicotinic acid, 96.5% purity; Sigma Aldrich, Madrid, Spain) concentrations of 0, 1, 10, 50, 100, 200, 300, 400 and 500 µmol L^{-1} were used. Absorbance of samples was measured with a spectrophotometer (Beckman DU-640, Fullerton, CA, USA) at 520 nm wavelength. The total content of the extracted protein in the ALS raw extract was measured using kit no. P5656 (Sigma-Aldrich) following the manufacturer´s instructions at 595 nm in the spectrophotometer.

GS activity was determined *in vitro* using 3 g (second aliquot) of the three wheat lines.[17] GS was purified from the raw extract.[22] GS activity was determined using technical grade glufosinate [(RS)-2-amino-4-(hydroxyl(methyl)phosphonoyl) butanoic acid], (95% purity, Sigma-Aldrich) at the following concentrations: 0, 6.25, 12.5, 25, 50, 100, 200 and 300 µmol L^{-1}. The absorbance of samples was measured in the spectrophotometer at 540 nm. The total content of the extracted protein in the GS experiment was measured using the colorimetric method described by Bradford.[23] The GS specific activity (nmol glutamine mg^{-1} protein h^{-1}) was determined in the absence of herbicide.

The experiments (ALS and GS assays) were repeated three times.

2.6 ALS sequencing

Leaf tissue (± 100 mg per sample) from the Rados and Helter lines and the Gazul cultivar was taken for DNA extraction using the Speed tools DNA Extraction Kit Cat Plant (Biotools B & M Labs. S.A., Madrid, Spain). The primer pair AHAS21Fwd/AHAS26Rev,[24] was used to amplify a 617-bp fragment. Polymerase chain reactions (PCR) were set up with Certamp complex enzyme mix (Biotools B & M Labs) following the manufacturer's instructions. PCR products (5 µL) were digested with the restriction enzyme *Msp*I (Invitrogen, Waltham, MA, USA) to identify three expected ALS alleles (*imi3*-, *imi1* and *imi2*-ALS genes from genomes A, B or D, respectively) of the catalytic subunit present in wheat varieties.[24] Both PCR and digestion products were resolved on 1% agarose gels and viewed under UV light. Ten PCR products of each allele and each wheat line/cultivar were sequenced using Sanger technology. ALS sequences were verified and assembled using SeqMan Pro 11 (DNASTAR, Madison, WI, USA) and Geneious 8.1.8 (Biomatters Ltd, Auckland, New Zealand) software, respectively, and compared with the ALS sequences of wheat accessions *imi1*-AY210407, *imi2*-AY210408 and *imi3*-AY273827 from GenBank.

2.7 Ammonia accumulation

Leaf discs (50 mg, 5 mm in diameter) from the youngest fully expanded leaves were placed in 1.5-mL tubes with 200 µL of different technical grade glufosinate concentrations (0, 6.25, 12.5, 25, 50, 100, 200 and 300 µmol L^{-1}) diluted in sucrose/Tween 20 medium. The tubes were sealed with two layers of micropore tape and placed in an incubation chamber at 18 °C under 150 mmol m^{-2} s^{-1} light intensity for 24 h, and 60% RH. Ammonia accumulation in R and S wheat plants was measured according to leaf disc method described by Dayan *et al.*[25] The absorbance of samples was measured with a spectrophotometer at 630 nm wavelength. The experiments were repeated twice, assessing three samples per glufosinate concentration (each with three replicates) of each wheat line. Ammonia content was determined using ammonium chloride as a standard (Sigma-Aldrich). Ammonia accumulation data were expressed as percentage of control.

2.8 Absorption and translocation

Absorption and translocation tests for each herbicide were carried out separately. Both imazamox and glufosinate labeled with ^{14}C were mixed with their respective commercial formulations (Pulsar® 40 and Finale®, respectively) to prepare a solution with a specific activity of 0.834 kBq µL^{-1}. The final concentrations corresponded to 40 g ai ha^{-1} of imazamox (+ 1.25 L ha^{-1} of methyl oleate/methyl palmitate adjuvant) and 750 g ai ha^{-1} of glufosinate in a 250 L ha^{-1} spray solution. Wheat plants with three to four true leaves were treated with a single 1-µL droplet of spray solution (0.834 kBq $plant^{-1}$) of ^{14}C-imazamox or ^{14}C-glufosinate, on the adaxial surface of the first or second youngest fully expanded leaf. Five plants per wheat line/cultivar were harvested at 3, 6, 12, 24, 48 and 96 h after treatment (HAT) for imazamox, and at 6, 12, 24, 48 and 72 HAT for glufosinate in a completely random design. The treated leaf was washed three times with 1 mL of water/acetone (1:1 v/v) to recover the non-absorbed ^{14}C-herbicide. The washing solution was mixed with 2 mL of scintillation liquid (Ultima Gold®, Packard BioScience BV, Groningen, The Netherlands) and radioactivity quantified by liquid scintillation spectrometry (LSS; Beckman LS 6500, Beckman Coulter Inc.). After washing for each indicated period, whole plants were removed from the pot, divided into treated leaf, rest of shoot plant and roots. The latter plant section was washed carefully with distilled water and excess moisture removed with a paper towel. Sections of each plant were stored individually in flexible combustion cones (Packard BioScience BV), dried in a stove at 60 °C for 1 week and combusted in a biological oxidizer (Packard Tri Carb 307; Packard Instrument Co., Downers Grove, IL, USA). The CO_2 released from the combustion was captured in 18 mL of a mix of Carbo-Sorb E and Permafluor (9:9 v/v) (Packard BioScience BV), and the radioactivity quantified by LSS. The percentage of each radiolabeled herbicide absorbed was expressed as [kBq in combusted tissue/(kBq in combusted tissue + kBq in leaf washes)] × 100.

The translocation of both ^{14}C-herbicides was also visualized. Wheat plants were treated and handled for the same periods indicated for each herbicide (three plants per wheat line/cultivar each time) using the same media as in the previous section. However, after removing the non-absorbed ^{14}C-herbicides, by washing the treated leaves with water/acetone (1:1, v/v) and the roots with distilled water, the plants were preserved whole, fixed on filter paper (25 x 12.5 cm) and dried at room temperature for 4 days. Samples were then placed on a phosphor plate for 6 h to visualize the ^{14}C-herbicide using a phosphor imager (Cyclone, Perkin-Elmer, Packard Bioscience BV).

2.9 Statistical analyses

Data for fresh weight reduction and enzyme activity assays were expressed as a percentage of the control, and subjected to non-linear regression analyses to determine the dose of imazamox and/or glufosinate needed to reduce the fresh weight (GR_{50}) and to inhibit enzyme (ALS or GS) activity (I_{50}) by 50% for each soft wheat line. Log-logistic three- or four-parameter models were used: $Y = c + \{(d-c)/[1 + (x/g)^b]\}$ or $Y = d/1 + (x/g)^b$):[26] where Y is the percentage fresh weight or the enzyme activity (ALS or GS) reduction with respect to the control; c is the lower limit; d is the

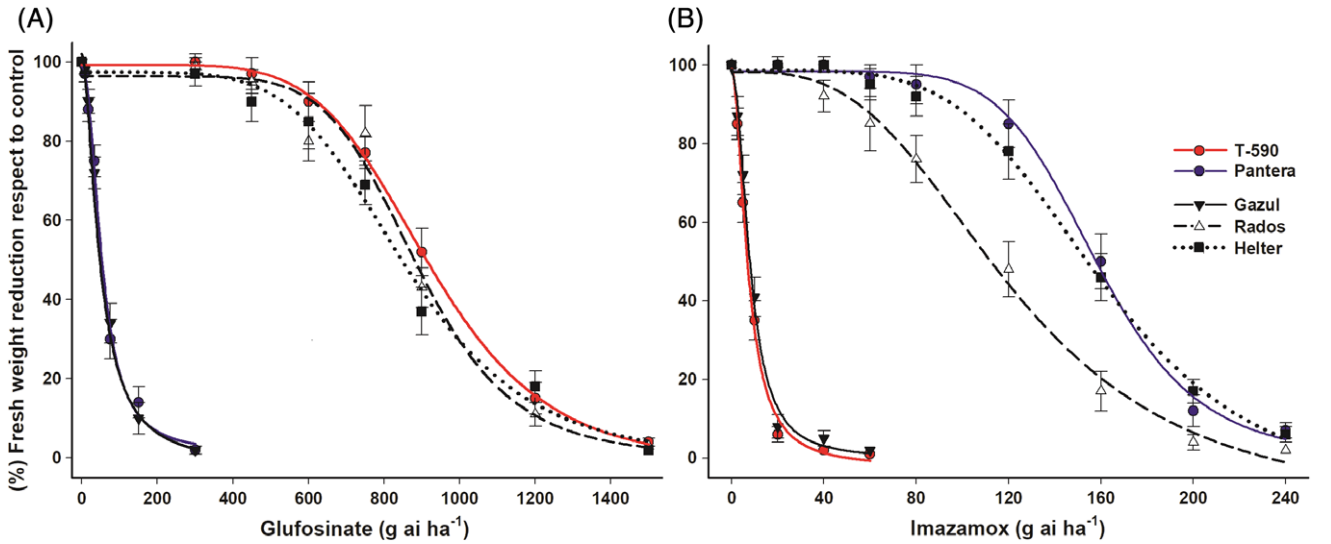

Figure 1. Dose–response curves of glufosinate (A) and imazamox (B) in resistant and susceptible soft wheat plants. The line T-590 (resistant to glufosinate) and the Pantera cultivar (resistant to imazamox) are parental to Rados and Helter, wheat lines with dual resistance to imazamox and glufosinate. The cultivar Gazul was used as a susceptible reference. Vertical bars ± SE ($n = 12$).

upper limit; *b* is the slope of the curve at the inflection point; *g* the herbicide dose at the inflection point; and *x* is the herbicide dose (independent variable). The three-parameter model assumes that the lower limit is zero. Regression analyses were conducted using the *drc* package in program R v. 3.2.5, and plotted using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA, USA). Resistance factors (RF = R/S) were computed as R-to-S GR_{50} or LD_{50} ratios.

Fresh weight reduction data corresponding to the interactions of imazamox (20, 40 and 60 g ai ha^{-1}) × glufosinate (450, 600 and 750 g ai ha^{-1}) were subjected to two-way analysis of variance (ANOVA), meanwhile, absorption and translocation of ^{14}C-herbicides data were subjected to one-way ANOVA. When required, the Tukey HSD test at 5% probability was used to separate means using the Statistix software v. 9.0 (Analytical Software, Tallahassee, FL, USA).

3 RESULTS

3.1 Dose–response

Dual resistance to imazamox and glufosinate was confirmed in the Rados and Helter lines. The R wheat lines, with similar glufosinate resistance to their resistant parent (T-590 line), were on average 18-fold more resistant than Gazul, the S cultivar (Fig. 1A). For imazamox, Rados and Helter lines were 15.1- and 19.8-fold more resistant than the S cultivar, respectively. Helter showed resistance to imazamox similar to that of its imazamox-resistant parent (cultivar Pantera), whereas that of the cultivar Rados was lower by 25% (Fig. 1B). As expected, parents resistant to a particular herbicide were susceptible to the other herbicide, showing ED_{50} values similar to the cultivar Gazul in each situation (Fig. 1, Table 1).

3.2 Interaction between herbicides

The GR_{50} values for the Rados and Helter lines, estimated for glufosinate and imazamox, decreased as the doses of the opposite herbicide increased (Table 2); therefore, dose–response curves were shifted to the left in all cases (Fig. 2). The GR_{50} values for glufosinate in wheat plants treated with 20 g ai ha^{-1} IMA were similar to those estimated in the glufosinate dose–response curves not treated with imazamox (Table 1). Resistance to glufosinate decreased ∼ 12% at 60 g ai ha^{-1} IMA. Resistance to imazamox decreased by between 6–9% and 20–26% in wheat plants treated with 450 and 600 g ai ha^{-1} GLU, respectively. However, this resistance decreased 38% and 50% for Rados and Helter, respectively, at +750 g ai ha^{-1} GLU (Table 2). Field rates minimum, intermediate and maximum of the opposite herbicide caused additive effects on the fresh weight reduction of wheat plants, except for the dose–response curves of imazamox with 750 g ai ha^{-1} GLU, where the effect was synergistic for both cultivars (Fig. 2B,D). The interaction 60 + 600 g ai ha^{-1} IMA + GLU, and any interaction of imazamox (20, 40, 60 g ai ha^{-1}) + 750 g ai ha^{-1} GLU caused the greatest fresh weight reduction rates in wheat plants (Fig. S1).

3.3 Foliar retention

Foliar retention (IMA + GLU) did not differ between wheat lines ($P = 0.1862$, DF = 2, $n = 21$). The herbicide solution retained was 228 ± 37, 214 ± 32 and 196 ± 27 µL g^{-1} dry weight for Helter and Rados lines and the Gazul cultivar, respectively.

3.4 Enzyme activity tests

The specific *in vitro* activities of the ALS enzyme for Rados and Helter lines and the Gazul cultivar were 279 ± 23, 294 ± 42 and 284 ± 28 nmol acetoin mg^{-1} protein h^{-1}, respectively, with no significant differences ($P = 0.2647$, DF = 2, $n = 72$). Imazamox inhibited the ALS activity in all cultivars as the concentrations increased (Fig. 3A). The amount of imazamox needed to inhibit the ALS enzyme activity of Gazul cultivar by 50% (I_{50}) was 11.8 µmol L^{-1}. The RF of Rados and Helter lines were 11.7 and 17.7, respectively, compared with the S cultivar (Table 3).

The specific *in vitro* activities of GS enzyme for Rados and Helter lines and the Gazul cultivar were 390 ± 46, 443 ± 33 and 421 ± 62 nmol of glutamine mg^{-1} protein h^{-1}, respectively, with no significant differences ($P = 0.3486$, DF = 2, $n = 72$). Glufosinate inhibited GS activity in all cultivars as the concentrations increased (Fig. 3B). The amount of glufosinate required to inhibit GS activity

Table 1. Parameters of the sigmoidal equations[a] used to estimate the dose (g ai ha^{-a}) of glufosinate and/or imazamox required to reduce the fresh weight by 50% (GR_{50}) in susceptible and resistant soft wheat plants

Cultivar/line	GR_{50} (95% CI)[b]	c	d	b	*P*	RF[c]
Glufosinate						
Gazul	47.4 (7.7)	2.2 ± 1.9	99.9 ± 1.5	1.7 ± 0.3	0.0036	–
Pantera	53.0 (6.2)	1.1 ± 0.8	98.8 ± 2.8	2.1 ± 0.3	0.0014	1.1
T-590	920.4 (72.6)	1.9 ± 2.2	99.3 ± 0.5	5.9 ± 0.3	< 0.0001	19.4
Rados	889.4 (46.3)	0.3 ± 0.1	96.4 ± 2.6	7.0 ± 2.2	< 0.0001	18.8
Helter	858.4 (54.8)	0.9 ± 0.4	97.5 ± 1.8	5.2 ± 1.0	0.0001	18.1
Imazamox						
Gazul	8.1 (0.7)	0.3 ± 0.2	97.9 ± 3.8	2.1 ± 0.3	0.0014	–
Pantera	168.4 (22.4)	0.1 ± 0.3	98.4 ± 1.1	7.2 ± 1.0	< 0.0001	20.8
T-590	7.1 (0.5)	2.3 ± 1.9	98.9 ± 3.0	1.9 ± 0.2	0.0009	0.9
Rados	122.5 (19.3)	1.2 ± 0.8	98.2 ± 1.6	3.1 ± 0.5	< 0.0001	15.1
Helter	160.6 (24.6)	0.9 ± 0.7	98.6 ± 0.9	4.7 ± 0.6	< 0.0001	19.8

[a] $Y = c + \{(d - c)/[1 + (x/g)^b]\}$ where *Y* is the percentage of fresh weight reduction with respect to control, *c* is the lower limit, *d* the upper limit, *b* the slope of the curve, *g* the herbicide dose at the inflection point (i.e. GR_{50}) and *x* is the herbicide dose.
[b] CI values are the 95% limits of confidence intervals ($n = 12$).
[c] Resistance factor (RF = GR_{50} of a resistant line or cultivar/GR_{50} of Gazul).

Table 2. Parameters of the sigmoidal equations[a] used to estimate the dose (g ai ha^{-a}) of glufosinate and/or imazamox, required to reduce the fresh weight by 50% (GR_{50}) on the Rados and Helter lines, in interaction with the minimum, intermediate and maximum field rates of the opposite herbicide

Cultivar/line	Herbicide interaction	GR_{50} (95% CI)[b]	c	d	b	*P*	DR[c]
	+ Imazamox (g ai ha^{-1})			Glufosinate[†]			
Rados (GR_{50} = 889.4)	20	894.2 (52.1)	0.1 ± 0.0	96.9 ± 1.7	7.3 ± 1.0	< 0.0001	1.00
	40	864.7 (43.8)	2.8 ± 1.3	97.7 ± 1.5	5.9 ± 0.6	< 0.0001	0.97
	60	783.7 (69.2)	3.7 ± 1.5	96.5 ± 4.4	4.8 ± 1.3	< 0.0001	0.88
Helter (GR_{50} = 858.4)	20	842.0 (30.8)	6.1 ± 4.1	96.0 ± 2.7	9.4 ± 2.1	< 0.0001	0.98
	40	806.7 (40.6)	3.2 ± 2.8	97.7 ± 1.7	6.4 ± 0.7	< 0.0001	0.93
	60	765.3 (47.3)	1.5 ± 1.3	95.0 ± 3.0	6.6 ± 1.3	< 0.0001	0.89
	+ Glufosinate (g ai ha^{-1})			Imazamox[‡]			
Rados (GR_{50} = 122.5)	450	112.0 (11.7)	–	96.7 ± 2.0	3.6 ± 0.5	< 0.0001	0.91
	600	98.7 (8.2)	–	94.4 ± 3.1	2.8 ± 0.5	< 0.0001	0.80
	750	76.4 (17.8)	–	95.3 ± 5.9	1.7 ± 0.5	0.0060	0.62
Helter (GR_{50} = 160.6)	450	151.0 (14.2)	–	97.7 ± 1.6	2.4 ± 0.3	0.0001	0.94
	600	119.2 (16.4)	–	94.5 ± 3.7	2.6 ± 0.6	0.0008	0.74
	750	81.9 (25.7)	–	97.9 ± 5.5	1.3 ± 0.4	0.0244	0.50

[a] $Y = c + \{(d - c)/[1 + (x/g)^b]\}$ (four parameters)† or $Y = d/1 + (x/g)^b$ (three parameters)‡: where *Y* is the percentage of fresh weight reduction with respect to the control, *c* is lower limit, *d* the upper limit, *b* the slope of the curve, *g* the herbicide concentration at the inflection point (i.e. GR_{50}), and *x* is the herbicide concentration. The three-parameter model assumes that the lower limit is zero.
[b] CI values are the 95% limits of confidence intervals ($n = 12$).
[c] Decrease in resistance (DR = GR_{50} of a wheat line treated with an additional dose of the opposite herbicide/GR_{50}, given in the first column, of the respective wheat line).

by 50% (I_{50}) ranged from 15.7 to 21.1 µmol L^{-1}. According to the 95% limits of confidence intervals (CI), there were no differences between R and S wheat lines (Table 3).

3.5 ALS sequencing

The amino acid sequences of Gazul cultivar (S) had the same consensus as the ALS sequences of wheat accessions from GenBank (*imi1*-AY210407, *imi2*-AY210408 and *imi3*-AY273827). The line Helter contained the Ser653–Asn mutation in the *imi1*- and *imi2*-ALS genes, whereas the line Rados contained the same mutation only in the *imi2*-ALS gene. The codon changes were AAC to AGC. No mutation was found in the *imi3* gene (Fig. 4).

3.6 Ammonia accumulation

The S and R lines accumulated ammonia at different levels. From 0 to 100 µmol L^{-1} of glufosinate, ammonia accumulation was similar. However, Gazul cultivar showed the highest ammonium accumulation (365 ± 88 µg of ammonia per g fresh weight) at 300 µmol L^{-1} of glufosinate. At this glufosinate concentration, Helter and Rados lines accumulated at least 60% less ammonia than Gazul cultivar (Fig. 5).

3.7 Absorption and translocation

More than 50% of ^{14}C-imazamox was absorbed within three HAT for all cultivars. Initially (3 to 12 HAT), the R lines absorbed greater amounts of herbicide than the S cultivar Gazul, but from 24 HAT

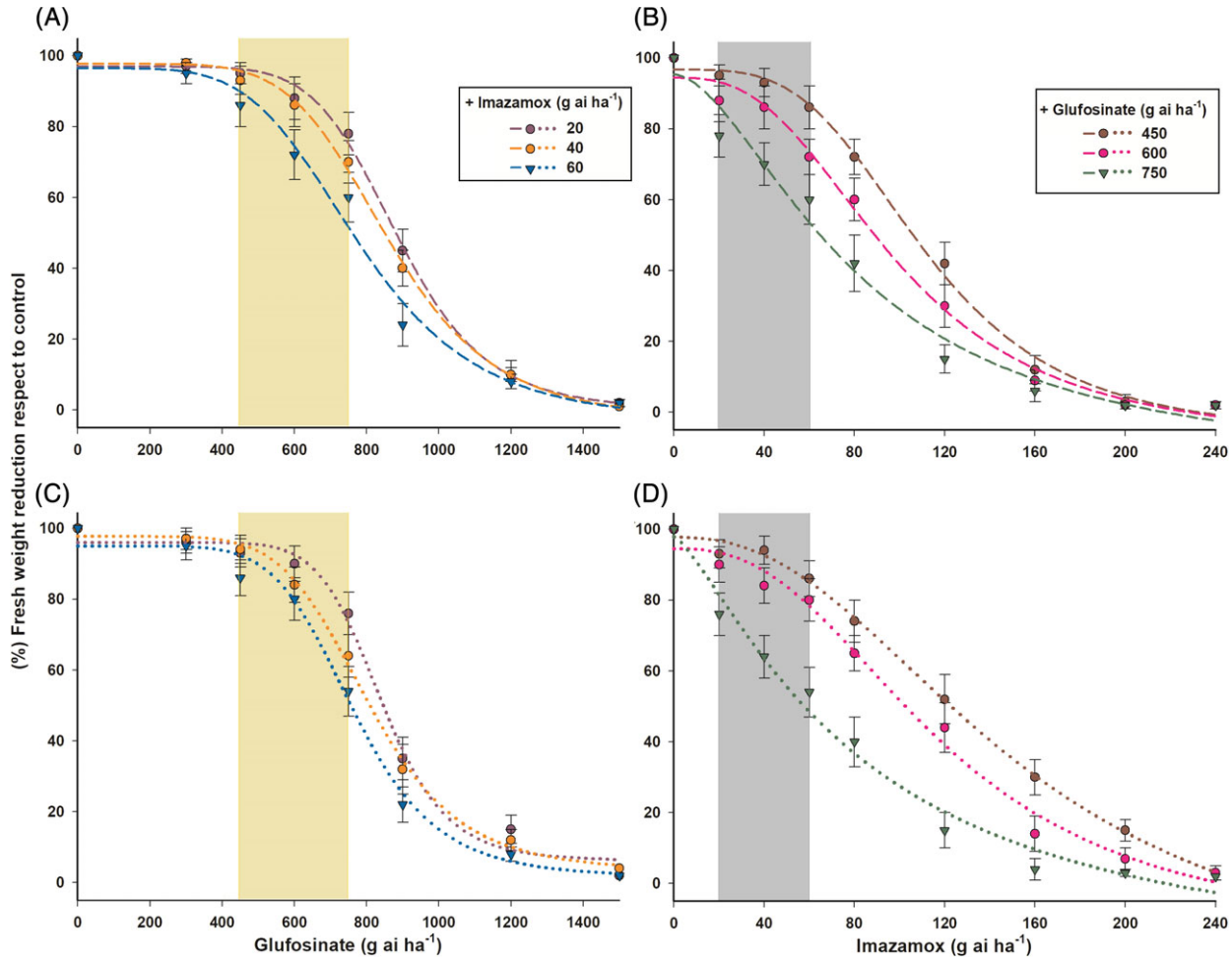

Figure 2. Dose–response curves of glufosinate (A, C) and imazamox (B, D) in interaction with the opposite herbicide on the wheat lines Rados (dashed lines) and Helter (dotted lines) with dual resistance to both herbicides. In all cases, glufosinate was applied 6 h after imazamox application. Boxes within the plots highlight the interaction of the minimum, intermediate and maximum recommended field rates between herbicides. Vertical bars ± SE ($n = 12$).

no differences were observed in the absorption between R and S wheat lines, with all showing > 90% ^{14}C-imazamox absorption (Fig. 6A). The R and S wheat plants showed high rates of ^{14}C-imazamox translocation from the treated leaf to the rest of the plant and roots. In the first 3 to 12 HAT, the ^{14}C-imazamox was mainly retained in the treated leaf, but from 24 HAT, extensive translocation to the rest of the plant and roots was observed. At 96 HAT, the ^{14}C-imazamox amount, quantified in each evaluated section of the plant, was similar between the wheat lines, ranging from 30% to 40% (Fig. 6B, Fig. S1).

Foliar ^{14}C-glufosinate absorption rates were < 40% of the total applied at the longest evaluated period (72 HAT). Differences in translocation rates were observed at 6, 24 and 48 HAT. The two R lines absorbed slightly more herbicide than the S cultivar (Fig. 6C). The R and S plants showed low translocation rates of ^{14}C-glufosinate with no difference among lines, with the majority of herbicide (> 75%) retained on the treated leaf. The amounts of ^{14}C-glufosinate translocated to the rest of the plant and roots were < 15% and 10%, respectively (Fig. 6D, Fig. S2).

4 DISCUSSION

Effective weed management without herbicide use is not currently viable in conventional intensive wheat production systems.[3] In this work, two new R wheat lines, with stacked traits conferring multiple resistance to imazamox and glufosinate, were characterized.

Rados and Helter lines presented acceptable levels of dual resistance to both herbicides. As per herbicide label, field rates of imazamox[27] and glufosinate[28] range from 20 to 60 and from 450 to 750 g ai ha^{-1}, respectively, depending on the cropping system, weed species and infestation level. The GR_{50} values of imazamox and glufosinate estimated for the R lines were above these recommended doses. Differences between the wheat R lines were due to their differential susceptibility to imazamox as discussed later.

The GR_{50} values for imazamox estimated for Rados and Helter were 122.5 and 160.6 g ai ha^{-1}, respectively. Imazamox doses (20, 40 or 60 g ai ha^{-1}) applied in the glufosinate dose–response curves were at least twofold lower than these values, and resistance to glufosinate of R wheat lines was reduced only slightly. By contrast, the ED_{50} values of glufosinate for both lines were close to the maximum dose of this herbicide applied in the imazamox dose–response curves; therefore, resistance to imazamox in Rados and Helter lines was drastically reduced when plants were treated with +750 g ai ha^{-1} glufosinate. Additive or synergistic effects of herbicides are desirable for weed control, but not for crop safety.[29] Although the sequential application of imazamox + glufosinate led to additive effects, the interactions between minimum and intermediate doses did not compromise the growth of Rados and

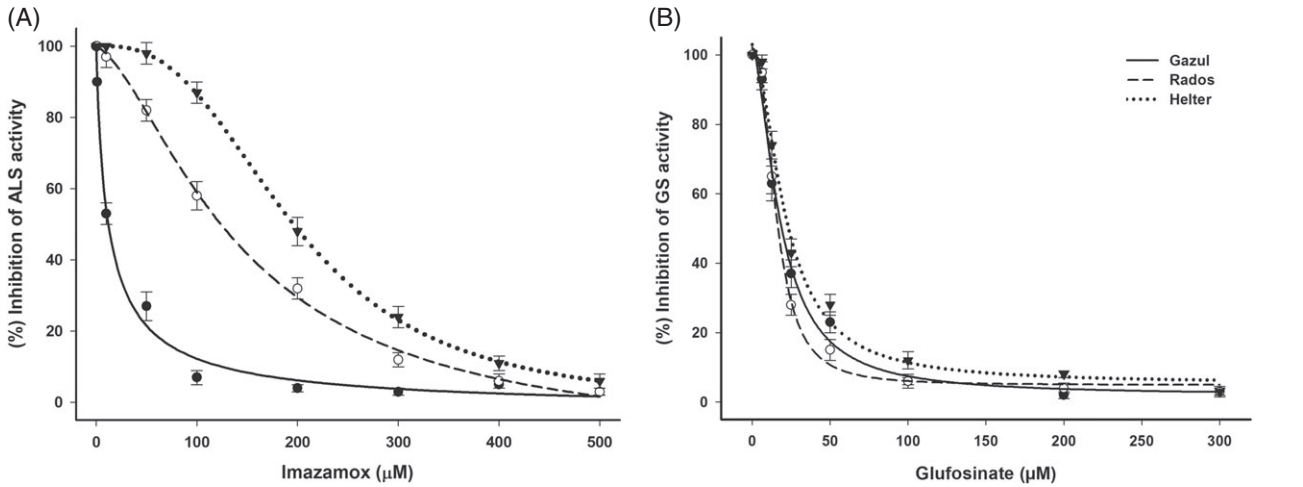

Figure 3. Log–logistic curves of enzymatic activity of the (A) acetolactate synthase (ALS) and (B) glutamine synthase (GS) in soft wheat plants of the lines Rados and Helter, with dual resistance to imazamox and glufosinate, and the susceptible cultivar Gazul. Vertical bars ± SE ($n = 3$).

Table 3. Parameters of the sigmoidal equations[a] used to estimate the concentrations (μM) of glufosinate and/or imazamox needed to inhibit the enzymatic activity of the ALS and GS by 50% (I_{50}), respectively, in susceptible and resistant soft wheat plants

Cultivar/line	I_{50} (95% CI)[b]	c	d	b	*P*	RF[c]
Glufosinate†						
Gazul	18.0 (4.7)	1.9 ± 1.1	102.2 ± 4.5	1.7 ± 0.3	0.0010	–
Rados	15.7 (5.1)	4.8 ± 1.8	101.8 ± 2.8	2.4 ± 0.3	< 0.0001	0.9
Helter	21.1 (7.2)	1.7 ± 0.7	103.0 ± 3.3	1.8 ± 0.3	0.0010	1.2
Imazamox‡						
Gazul	11.8 (3.4)	–	100.0 ± 3.6	0.9 ± 0.1	0.0039	–
Rados	137.6 (15.1)	–	99.9 ± 1.1	1.6 ± 0.1	< 0.0001	11.7
Helter	208.7 (19.8)	–	99.8 ± 0.3	2.8 ± 0.1	< 0.0001	17.7

[a] $Y = c + \{(d - c)/[1 + (x/g)^b]\}$ (four parameters)† or $Y = d/1 + (x/g)^b$ (three parameters)‡: where *Y* is the percentage of enzyme activity with respect to the control, *c* is the lower limit, *d* the upper limit, *b* the slope of the curve, *g* the herbicide concentration at the inflection point (i.e. I_{50}), and *x* is the herbicide concentration. The three-parameter model assumes that the lower limit is zero.
[b] CI values are the 95% limits of confidence intervals ($n = 3$).
[c] Resistance factor ($RF = I_{50}R/I_{50}S$).

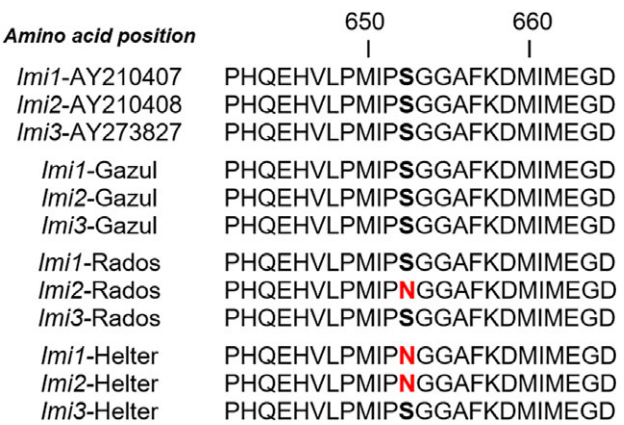

Figure 4. Partial alignment of deduced amino acid sequence of the *imi*-ALS genes sequences in soft wheat plants of the lines Rados and Helter, with dual resistance to imazamox and glufosinate, and the susceptible cultivar Gazul. Highlighted letters indicate the Ser653 position corresponding to the point mutation associated with conferring of resistance to imazamox. Red letters indicate a codon change from AAC (serine = S) to AGC (asparagine = N). Amino acid number based on the *A. thaliana* ALS sequence (GenBank accession no.: AY042819).

Helter plants. Thus, combinations of 20 + 450, 20 + 600, 40 + 450, 40 + 600 and 60 + 450 g ai ha^{-1} IMA + GLU may be applied during the same cropping season. However, the maximum dose of glufosinate (750 g ai ha^{-1}) had synergistic effects in combination with imazamox; therefore, application of this dose it is not recommended. In addition, Helter and Rados lines were more resistant when exposed to these herbicides separately, which could facilitate the rotation of herbicides between cropping seasons. This practice and the correct adoption of HR crops may contribute to mitigating the increasing problems with HR weeds.[7]

R and S wheat plants presented similar foliar retention of herbicide solution, suggesting that this parameter did not play an important role in the dual resistance to imazamox and glufosinate in Rados and Helter lines. In addition, differences in the foliar retention of imazamox solution were observed among wheat cultivars/lines, S or R, but this did not influence the resistance levels.[30]

The greater I_{50} value of ALS enzyme in the R wheat lines, Rados and Helter, indicates alterations in the target site. This suggests that the ALS-*imi1* and ALS-*imi2* resistant genes, with mutation Ser653–Asn from the imazamox-resistant cultivar Pantera (parent),[15] were transferred to the R wheat lines. Differences in imazamox susceptibility between the R lines, as demonstrated in

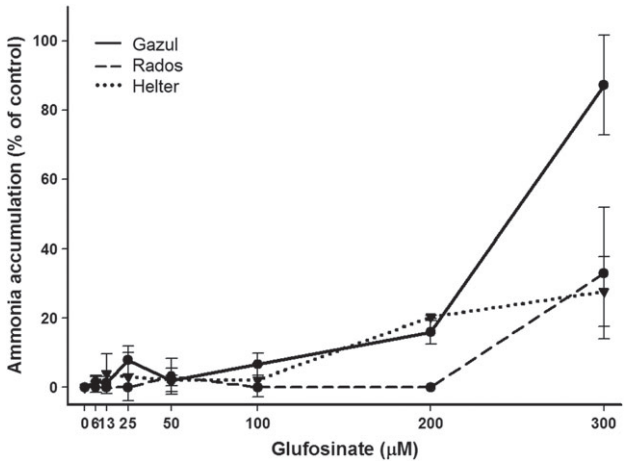

Figure 5. Accumulation of ammonia (%) at different concentrations (μM) of glufosinate in soft wheat plants of the lines Rados and Helter, with dual resistance to imazamox and glufosinate, and the susceptible cultivar Gazul. Vertical bars ± SE ($n = 3$).

ALS gene sequencing, were due to the Helter line acquiring both resistant genes (ALS-*imi1* and ALS-*imi2*) from the Pantera cultivar, whereas the Rados line acquired only one (ALS-*imi2*). Generally, Clearfield® crops carry only the mutation in the ALS-*imi2* gene, which is enough to confer resistance to imazamox[16,31] at recommended field doses (40 g ia ha^{-1}). Similar differences in imazamox susceptibility were observed in Clearfield® wheat cultivars[14–16] and other Clearfield® crops,[31] carrying the single and/or double mutation in the *imi1*-ALS and *imi2*-ALS genes, respectively. However, the similar susceptibility of the GS to glufosinate in wheat plants R and S, suggested that there are no mutations in the GS gene involved in the resistance of Helter and Rados lines to this herbicide.

The similar specific ALS and GS activities of the wheat lines (both R and S) in the absence of herbicides (imazamox and glufosinate, respectively), suggested that overexpression of both ALS and GS enzymes was not involved in the multiple resistance to imazamox and glufosinate of Rados and Helter lines. The large accumulation of ammonia in Gazul, the S cultivar, showed a rapid inhibition of GS activity by glufosinate in this cultivar. Ammonia accumulation

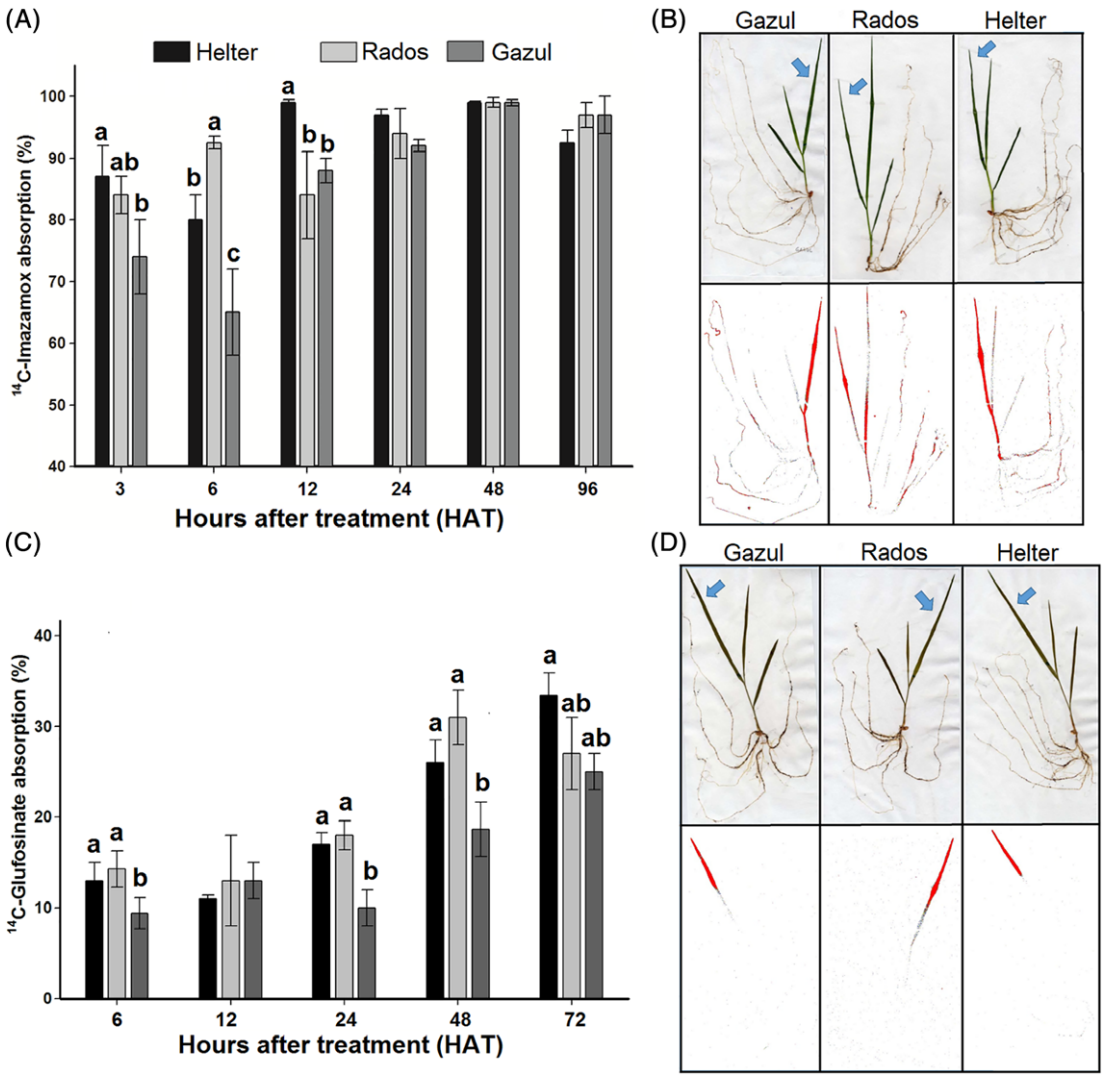

Figure 6. Absorption of ^{14}C-imazamox (A) and ^{14}C-glufosinate (C) from 3 to 96 h after treatment (HAT) and from 6 to 72 HAT, respectively, in soft wheat plants of the lines Rados and Helter, with dual resistance to imazamox and glufosinate, and the susceptible cultivar Gazul. Vertical bars represent ± SE ($n = 5$). Digital and autoradiograph images of ^{14}C-imazamox (B) and ^{14}C-glufosinate (D) translocation at 96 HAT. The red color indicates a higher concentration of ^{14}C-herbicide. Arrows indicate the treated leaf.

is an indicator of the susceptibility of the GS enzyme to glufosinate toxicity.[32] The lower accumulation of ammonia in R-wheat lines indicates that GS activity was not impaired by glufosinate.[13] This was presumably due to rapid acetylation of the glufosinate to *N*-acetyl-glufosinate (non-toxic compound) mediated by the PAT enzyme. This enzyme caused low ammonia accumulation in glufosinate-resistant cotton[13] and rice[33] that carried the *bar* gene. Once this gene is responsible for encoding the PAT enzyme, and considering the accumulation of ammonia as an indicator of resistance in glufosinate-resistant crops, we concluded that the Helter and Rados lines acquired and conserved the *bar* gene from their glufosinate-resistant parent, the T-590 line,[17,18] after crossing with the cultivar Pantera.

The R and S wheat lines showed high absorption and translocation patterns of ^{14}C-imazamox. During the first HAT (3 to 12 HAT), there were differences in absorption between wheat lines. This parameter was higher in R than in S wheat plants during this period, but translocation of ^{14}C-imazamox was similar between them during the evaluation period (3 to 96 HAT). Reduced absorption and translocation may have an important role in the resistance to other herbicides such as glyphosate,[34,35] but in the resistance to ALS-inhibiting herbicides this role is secondary.[11] This suggests that these non-target site mechanisms were not involved in the resistance to imazamox of these R wheat lines.

Unlike imazamox, the absorption and translocation patterns of ^{14}C-glufosinate were lower, because this herbicide has low or no mobility due to its rapid phytotoxicity in the source leaf tissue.[28,36] This explain the low amounts of glufosinate absorbed and translocated in the R and S wheat plants. The limited translocation of ^{14}C-glufosinate may be a consequence of the combined effects of ammonia accumulation, reduced carbon accumulation and glutamine depletion.[17,32,37] These processes occurred rapidly in S plants, killing their leaf tissues, and explaining why these plants show lower absorption and translocation rates for ^{14}C-glufosinate in comparison with R plants. Therefore, the absorption and translocation of these herbicides were not involved in the resistance to glufosinate of the R wheat lines.

5 CONCLUSIONS

The R wheat lines, Rados and Helter, showed high levels of resistance to imazamox and glufosinate. Stacked traits conferring this dual herbicide resistance were obtained from their respective glufosinate- and imazamox-resistant parents.

Sequential application of low or intermediate field rates of imazamox + glufosinate during the same cropping season does not compromise plant growth in R wheat. However, the resistance of Rados and Helter lines is higher when exposed to these herbicides independently, facilitating rotation of herbicides. Therefore, these R lines are an effective weed management tool for wheat production, and may also contribute to mitigating the increasing problem of HR weeds.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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