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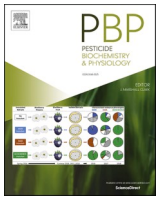

First case of triple resistance to EPSPS, ALS, and synthetic auxin herbicides in *Bassia scoparia* (L.) Voss in Europe

Germán Mora [a,*], Miriam Gil-Monreal [b], Maria Dolores Osuna [c], Vijaya Bhaskar Alwarnaidu Vijayarajan [d], José María Montull [a], Josep María Llenes [e], Jordi Recasens [a], Alicia Cirujeda [f,g], Ana Isabel Marí [f], Joel Torra [a,*]

[a] Department of Agricultural and Forest Sciences and Engineering, University of Lleida – Agrotecnio CERCA Centre, Lleida, Spain
[b] Institute for Multidisciplinary Research in Applied Biology (IMAB), Universidad Pública de Navarra (UPNA), Campus de Arrosadia, Pamplona, Spain
[c] Extremadura Scientific and Technological Research Center (CICYTEX), Plant Protection Department, Badajoz, Spain
[d] Crop Science Department, Teagasc Oak Park Research Centre, Carlow, Ireland
[e] Weed Science – Unit of the Plant Protection Service, DARP, Generalitat de Catalunya, Lleida, Spain
[f] Departamento de Sistemas Agrícolas, Forestales y Medio Ambiente (SAFMA-CITA), Zaragoza, Spain
[g] Instituto Agroalimentario de Aragón—IA2 (CITA—Universidad de Zaragoza), Zaragoza, Spain

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ABSTRACT

Bassia scoparia (L.) Voss has evolved resistance to five herbicide modes of action (MoAs) worldwide, including multiple resistance to up to four MoAs. Seeds were collected from a putatively resistant *B. scoparia* population (GUI-R) that survived successive herbicide applications of synthetic auxins, acetolactate synthase (ALS), and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitors in a no-till winter cereal field in Catalonia, Spain, in 2022, to assess resistance levels and mechanisms. Dose-response assays confirmed that GUI-R was 2-, 340-, 42.7-, and 60-fold more resistant to glyphosate, thifensulfuron, MCPA, and 2,4-D, respectively, based on plant weight, and 3.2-, 123-, 57.9-, and 32-fold more resistant based on plant survival. GUI-R showed cross-resistance to imazamox (46 % survival), but not to dicamba or fluroxypyr (100 % mortality), at the label rate. Preliminary studies using malathion pre-treatment, a cytochrome P450 inhibitor, reversed 2,4-D resistance in GUI-R at the label rate, resulting in a 97 % reduction in biomass. Molecular studies revealed that GUI-R has 4.9 additional copies of the *EPSPS:ALS* gene, with no known mutations and less shikimate accumulation than the susceptible population. *ALS* gene sequencing identified the Pro197Ser, Pro197Leu, and Trp574Leu mutations, along with a combined Pro197Ser + Trp574Leu mutation. In conclusion, *EPSPS* gene amplification and ALS mutations confer target-site resistance to glyphosate, thifensulfuron and imazamox in GUI-R. Resistance to 2,4-D and MCPA is probably driven by P450-mediated non-target-site resistance and further research is necessary to confirm mechanisms. This biotype represents the first case of glyphosate resistance in Europe for the species, as well as the first triple resistance.

1. Introduction

Bassia scoparia (L.) Voss., formerly *Kochia scoparia* (L.) Schrad., is a diploid (2n = 18) annual broadleaf weed of the *Amaranthaceae* family, native to Eurasia (Friesen et al., 2009). Its C_4 photosynthetic pathway provides adaptive advantages in various soil types and climatic conditions, including saline soils and drought stress (Friesen et al., 2009; Kumar and Jha, 2017; Yadav et al., 2023). Its competitiveness with various crops can potentially reduce crop yield by 7–68 %, making herbicide-based weed management a high priority (Geddes and Sharpe, 2022).

Since 1976, when resistance to atrazine was first reported (a photosystem II inhibitor, PSII/HRAC Group 5), which is now banned in the European Union, *B. scoparia* has developed resistance to five different herbicide modes of action (MoAs). There have been 45 cases of single resistance, primarily to acetolactate synthase (ALS/HRAC Group 2), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS/HRAC Group 9), PSII inhibitors, and more recently, to protoporphyrinogen oxidase

* Corresponding authors.
E-mail address: german.mora@udl.cat (G. Mora).

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inhibitors (PPO/HRAC Group 14) (Geddes et al., 2025). Resistance to synthetic auxins (HRAC Group 4) has been reported less frequently, along with 11 cases of multiple resistance to ALS (sulfonylureas) and EPSPS inhibitors (Beckie et al., 2013; Heap, 2025; Kumar et al., 2015; Rana and Jhala, 2016). Additionally, populations resistant to three (HRAC 2, 4, and 9) and four (HRAC 2, 4, 5, and 9) MoAs have been documented (Beckie et al., 2019b; Varanasi et al., 2015). Notably, 23 % of resistance reports are associated with populations found along roadsides or railway tracks (Heap, 2025), including the first European case of resistance to both ALS and PSII inhibitors in this species (Salava and Chodová, 2007).

Herbicide resistance in *B. scoparia* is primarily attributed to target-site resistance (TSR) mechanisms (Kumar et al., 2019a), with transposable elements (TEs) playing a crucial role in enhancing genetic variability, amplifying resistance genes, and enabling rapid adaptation to stressful conditions (Dyer, 2018; Sen et al., 2025). ALS-inhibiting herbicides act by blocking acetolactate synthase, an enzyme essential in the biosynthetic pathway of branched-chain amino acids (Tranel and Wright, 2002). The most common resistance mechanism to ALS inhibitors in weeds involves mutations in the nine conserved domains of the *ALS* gene (Fang et al., 2022; Murphy and Tranel, 2019). In *B. scoparia*, mutations at ALS positions Pro197, Trp574, and Asp376 have been identified, with Pro197 being the most frequently mutated. Different amino acid substitutions at Pro197 confer cross-resistance to sulfonylurea (SU, e.g., thifensulfuron) and triazolopyrimidine (TP), whereas Trp574 confers cross-resistance to SU, TP, and imidazolinones (IMI, e.g., imazamox). In addition, Asp376 is associated with SU resistance (Foes et al., 1999; Heap, 2025; Warwick et al., 2008). Additionally, non-target-site resistance (NTSR) mechanisms, mediated by cytochrome P450 (P450) and glutathione S-transferase (GST) enzymes, contribute to metabolic herbicide detoxification, leading to cross-resistance to ALS-inhibiting herbicides (Rigon et al., 2020). The herbicide glyphosate inhibits the EPSPS enzyme, blocking the shikimate biosynthetic pathway, which is essential for the production of aromatic amino acids in plants (Duke, 2020). Glyphosate resistance in *B. scoparia* is primarily attributed to an increased copy number of the *EPSPS* gene, mediated by a TE containing an *FHY3/FAR1* gene (Gaines et al., 2016; Patterson et al., 2019). Synthetic auxin herbicides, such as 2,4-D and MCPA (phenoxy-carboxylates), dicamba (benzoates), and fluroxypyr (pyridyloxy-carboxylates), mimic the action of indole-3-acetic acid, causing excessive hormonal accumulation, epinasty, and uncontrolled tissue elongation (Grossmann, 2010). In *B. scoparia,* resistance to synthetic auxins is primarily driven by different NTSR mechanisms, including reduced translocation, enhanced absorption, and increased metabolism, with a secondary contribution from TSR mutations (Moreno-Serrano et al., 2024; Todd et al., 2020).

B. scoparia is an outcrossing tumbleweed-forming species that produces approximately 100,000 seeds per plant, with up to 90 % of its seeds dispersed within a 1000 m radius, thereby spreading its resistance genes (Beckie et al., 2016). Previous studies on pollen-mediated gene flow (PMGF) have demonstrated the short-range dispersal of glyphosate resistance, with outcrossing rates of 5.3 % at 4.1 m, 7.5 % at 2.5 m, and as high as 13.1 % at 1.5 m in SU-resistant populations (Beckie et al., 2016; Stallings et al., 1995). This outcrossing nature of *B. scoparia* enhances PMGF, contributing to the rapid spread and establishment of resistance alleles (Beckie et al., 2019a; Beckie and Martin, 2021; Jhala et al., 2021). Although species seed bank longevity in soil is typically less than two years (Dille et al., 2017), plants trapped in fences or barriers can retain up to 18.5 % of their seeds, forming aerial seed banks that facilitate winter survival and the emergence of new cohorts (Geddes and Pittman, 2023).

In Spain, *B. scoparia* has become increasingly problematic in reduced tillage systems, particularly in orchards, woody perennial crops, and no-till winter cereals (Montull and Torra, 2023; Recasens et al., 2018). The most affected areas are located in the Ebro Valley (Catalonia and Aragón), Albacete, and, to a lesser extent, La Rioja. In conventional orchard management systems, weed control typically involves glyphosate applications (alone or tank-mixed with MCPA) along the crop row, combined with mechanical control or cover crops in the inter-row space. In winter cereals, glyphosate is commonly applied pre-plant, followed by ALS-SU in post-emergence (spring), and tank mixtures of glyphosate and 2,4-D during the post-harvest period (July–August). The area at risk of *B. scoparia* infestation includes 671,941 ha—mainly olive, vineyard, and almond orchards—and 1,409,194 ha of winter cereals (Ministerio de Agricultura, Pesca y Alimentación MAPA, 2024).

In the summer of 2022, cereal growers in northeastern Spain reported multiple herbicide control failures in *B. scoparia,* although there was no evidence to support this claim until this study was conducted. The objectives of this study were to confirm and characterize the resistance levels and mechanisms of EPSPS, ALS, and synthetic auxin herbicides in a putatively resistant population from a no-till winter cereal field in Catalonia, Spain.

2. Materials and methods

2.1. Plant material

In the fall of 2022, seeds from a putatively resistant *B. scoparia* population (GUI-R) were collected from approximately twenty plants that had survived successive applications of synthetic auxin, ALS-inhibiting, and EPSPS-inhibiting herbicides in a no-till winter cereal field located in Guissona, Catalonia, Spain. As a susceptible reference population (A17-S), *B. scoparia* seeds were obtained from the Botanical Garden of Arable Weed Species at CITA, Zaragoza (Aragon, Spain) without any history of herbicide applications.

Seeds of GUI-R and A17-S were sown in trays filled with moist peat in spring 2023. They were then placed in a growth chamber set to 28/22 °C (day/night) with a 12-h photoperiod and a photosynthetic photon flux density of 850 μmol m^{-2} s^{-1}. Five days after germination, the seedlings were transplanted into 392 cm^3 pots containing a 1:1 mixture of loamy soil and peat. The plants were maintained in a greenhouse at a constant temperature of 30 °C and a 16-h photoperiod, with regular irrigation, for one week until they reached a height of 5–6 cm, at which point herbicide treatments were applied.

2.2. Evaluated herbicides

Post-emergence herbicides registered for use in winter cereal crops were applied, representing three distinct MoAs: EPSPS (glyphosate) and ALS inhibitors (thifensulfuron and imazamox), and synthetic auxins (2,4-D, MCPA, fluroxypyr, and dicamba) (Table 1), along with a non-ionic adjuvant (0.5 L ha^{-1}, Dash® 90, FMC, 90 % *w/v*). Treatments were conducted using a static precision bench sprayer calibrated to deliver a spray volume of 200 L ha^{-1} at a pressure of 215 kPa. A low-drift 110° flat-fan nozzle (Low Drift, ISO LD-02-110-CT, yellow, HARDI®) was used for herbicide application.

2.3. Whole-plant dose-response bioassays

Three independent experiments, one per MoA, were conducted in the summer of 2023 in the greenhouse at the University of Lleida (41° 37′N, 0° 35′E). Six to seven increasing doses of EPSPS inhibitor (glyphosate), ALS inhibitor (thifensulfuron), and synthetic auxin (2,4-D and MCPA) herbicides were evaluated. The number of doses was adjusted according to preliminary sensitivity assays (data no shown) for each herbicide–population combination and the anticipated level of resistance (Table 1). For each population, ten replicates per dose were analyzed in a completely randomized design (one plant per pot = one replicate). The entire experiment was repeated twice. Twenty-eight days after treatment (DAT), plant survival percentage and fresh weight reduction relative to the untreated control were assessed. A modified visual injury rating scale (0–100 %) based on Cranston et al. (2001) was used to

Table 1
Characteristics of the evaluated herbicides and doses used in whole-plant dose–response assays.

MoA[a]	Herbicide	Population	Rates (g ae ha^{-1})
EPSPS	glyphosate (Roundup plus®, 36 % *w/v*, Bayer)	GUI-R	0 100 200 400 800 1200 1600
		A17-S	0 25 50 100 200 400 800[c]
ALS	thifensulfuron[b] (Harmony®, 50 % *w/w*, FMC)	GUI-R	0 7.5 15 30 60 120 240 480
		A17-S	0 0.23 0.47 0.94 1.88 3.75 7.5[c]
	imazamox[b] (Pulsar®, 40 %w/v, BASF)	GUI-R	0 40
		A17-S	0 40[c]
Auxin mimics	2,4-D (Esteron®, 60 % *w/v*, Corteva)	GUI-R	0 150 300 600 900 1200 1800
		A17-S	0 9.4 18.8 37.5 75 150 300 600[c]
	MCPA (Arges®, 70 % *w/v*, Karyon)	GUI-R	0 300 600 1200 2400 4800 9600 19,200
		A17-S	0 9.4 18.8 37.5 75 150 300 600[c]
	dicamba (Banvel-D®, 48 % *w/v*, Syngenta)	GUI-R – A17-S	0 144 288[c] 1152
	fluroxypyr-methyl (Hudson®, 20 % *w/v*, Key S.A)	GUI-R – A17-S	0 100 200[c] 800

[a] Mode of action.
[b] Doses are expressed in g ai ha^{-1}.
[c] Field rate.

evaluate plant damage following synthetic auxin treatments. A plant was considered alive or resistant if it exhibited up to 40 % injury, retained an active growing point, and maintained a leaf angle of 90° relative to the stem (Supplementary material. Spreadsheet 1).

2.4. Cross-resistance herbicides and 2,4-D metabolism

Three increasing doses of the synthetic auxins dicamba and fluroxypyr, along with the ALS inhibitor imazamox (applied at the recommended label rate), were evaluated for efficacy and potential cross-resistance (Table 1). Treatments were arranged in a completely randomized design, with 16 plants per dose and population. Each plant was treated as an independent experimental replicate, and untreated controls were included for comparison. Additionally, the involvement of P450-mediated metabolism in 2,4-D resistance was evaluated by spraying plants ($n = 12$ for GUI-R and $n = 6$ for A17-S) with 1000 g ai ha^{-1} of malathion at least three hours prior to treatment with 2,4-D at the label dose rate (600 g ae ha^{-1}). Untreated controls, plants treated with malathion +2,4-D and plants treated with 2,4-D alone were included. Growth parameters, herbicide application, and evaluation criteria for these assays were as previously described (sections 2.1, 2.2 and 2.3).

2.5. Shikimate assay

Shikimate accumulation following glyphosate treatment in GUI-R and A17-S was assessed as described in Fernández-Escalada et al. (2016). Six leaf discs per plant (4 mm in diameter) were collected from the youngest leaves of each population using a punch with plunger (KAI Medical, Solingen, Germany). Eight replicates (one plant = one replicate) and a flat-bottom microplate (Anicrin SRL, Scorzè, Italy) were used per population. Shikimate was extracted and quantified spectrophotometrically at 380 nm. The experiment was repeated twice.

2.6. EPSPS copy number determination and EPSPS and ALS gene sequencing

2.6.1. DNA extraction

Leaf tissue (~100 mg) was collected from the GUI-R population that survived glyphosate and thifensulfuron treatments ($n = 11$ or 12 plants), as well as from the susceptible A17-S population ($n = 10$ or 12 plants). Samples were stored at −80 °C until genomic DNA (gDNA) was extracted. Tissue samples were ground in liquid nitrogen, and about 50 mg of each sample was used for gDNA extraction using the Speedtools Plant DNA Extraction Kit (Biotools B&M Labs S.A., Valle de Tobalina, Madrid, Spain). The concentration of each sample was measured in a NANODROP Thermoscientific spectrophotometer (ThermoFisher, NanoDrop Products, Wilmington, DE). The quality was assessed by 1 % agarose gel electrophoresis. gDNA was stored at −20 °C.

2.6.2. EPSPS copy number determination

Quantitative real-time polymerase chain reaction (qPCR) was performed on the GUI-R ($n = 15$ plants) and A17-S (n = 10 plants) populations to evaluate the relative copy number of the *EPSPS* gene. NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) software were used to confirm the stability of two endogenous low-copy control genes, *ALS* and *carbamoylphosphate synthetase* (*CPS*) (Ma et al., 2013). The primers used were: EPSPS_F (5′-GGCCAAAAGGGCAATCGTGGAG-3′), EPSPS_R (5′-CATTGCCGTTCCCGCGTTTCC-3′), ALS_F (5′-ATGCAGACAATGTTGGATAC-3′), ALS_R (5′-TCAACCATCGATACGAACAT-3′) (Wiersma et al., 2015), CPS_F (5′-GACCTTGACTGACAGGGAATAC-3′), and CPS_R (5′-AACATTAGACCCACCACACTC-3′). Primers targeting the *CPS large subunit* gene of *B. scoparia*, based on the *Bs.00 g131420* gene sequence obtained from the WeedPedia database, a platform hosting genomic data generated by the International Weed Genomics Consortium and curated by KeyGene (KeyGene N.V., Wageningen, The Netherlands), were designed using the PrimerQuestTM tool (Integrated DNA Technologies, Inc., IOWA, USA). Each qPCR reaction contained a total volume of 20 μL, including 10 μL of SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), 0.6 μL of each forward and reverse primer, 4.8 μL of water, and 4 μL of genomic DNA (2.5 ng/μL). The cycling conditions were as follows: 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s and 62 °C for 1 min, and melting curve analysis from 65 °C to 95 °C with a heating rate of 0.1 °C s^{-1}. Water was used as the negative control. The relative *EPSPS* copy number was calculated using the 2ΔCt comparative method ($\Delta Ct = Ct^{EPSPS} - Ct^{ALS}$) (Gaines et al., 2010).

2.6.3. PCR and sequencing of the EPSPS gene

PCR amplification was performed in a 50 μL reaction mixture containing 25 μL of Premix Taq™ DNA Polymerase (Takara Bio Group, reference R004A), 2.5 μL of each forward and reverse primer (10 μM), 15.5 μL of nuclease-free water (Thermo Fisher Scientific, reference 0581), and 4.5 μL of gDNA (0.05 μg/μL). Using the primers 5′-ATGTTGGACGCTCAGAACT-3′ and 5′-TGAATTTCCTCCAGCAACGGC-3′, a 200-bp fragment of the conserved TAP region of the *EPSPS* gene, encompassing the codons for residues Thr102, Ala103, and Pro106, was amplified (Wiersma et al., 2015). The PCR cycling conditions were as follows: initial denaturation at 98 °C for 1 min, followed by 40 cycles of 98 °C for 10 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 4 min, with samples held at 4 °C thereafter. The quality and quantity of the PCR products were verified by 2 % agarose gel electrophoresis and microplate reader analysis (wavelength: 320 nm). Samples with gDNA concentrations >20 ng/μL were sequenced using the Sanger method (3730xl DNA Analyzer, Thermo Fisher Scientific) by Lab STAB VIDA (Caparica, Portugal). Sequences were visualized and aligned using Chromas 2.6.6 and MEGA 11.0.13 software, with accession number KJ374721.1 (GenBank) as the reference sequence.

2.6.4. PCR and sequencing of the ALS gene

For *ALS* gene sequencing study, two pairs of primers were used. One pair, BE4F (5′-GGGTGGAAAATCTCCCTGTT-3′)/ BE4R (5′-CGAGCAG-CAGGAATATCACA-3′) was designed by using the PRIMER 3 PLUS program to amplify the BE region of the *ALS* gene, while the region CAD of gene was amplified using the pair of primers KGenFor (5′-CGGGCCGTGTTGGTGTCT-3′) and RuTh-R-2 (5′- GACA-CATGGGGTTGCTTATTCTTC-3′) (Beckie et al., 2011). The mixture of reagents used to carry out the PCR was 0.75 μL of each primer (10 pmol μL^{-1}), 1.6 μL of the dNTP mixture (2.5 mM), 2 μL of 10× buffer, 0.2 μL of Taq polymerase (DreamTaq DNA, Thermo Scientific, 5 U μL^{-1}), and PCR water to a final volume of 20 μL.

The PCR cycling conditions for both pairs of primers were similar: 95 °C for 5 min (×1), followed by 35 cycles of 95 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min, with the samples held at 4 °C thereafter. For the BE1-F/BE-R primer pair, the PCR cycle consisted of 95 °C for 5 min (×1), followed by 35 cycles of 95 °C for 30 s, 61 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min, with samples held at 4 °C thereafter. PCR products were processed as described in the glyphosate section 2.7.2. The reference sequence used for alignment was EU517490.1 (GenBank).

2.7. Statistical analysis

Data from the dose-response experiments were analyzed using SigmaPlot 12.0 software (Systat Software, Inc., San José, CA, USA). Fresh weight and survival data were used to estimate the herbicide dose required to cause a 50 % or 90 % reduction in growth (GR_{50} and GR_{90}) or mortality (LD_{50} and LD_{90}) using a four-parameter non-linear regression model (Seefeldt et al., 1995) based on the following equation:

$$y = c + \frac{d - c}{1 + EXP\,[b(\log(x) - \log(LD_{50}\ or\ GR_{50}))]}$$

where c is the lower limit (adjusted to 0), d is the upper limit (adjusted to 100), b is the slope around LD_{50} or GR_{50}, x is the herbicide dose, which is the independent variable and y is the response (i.e. fresh weight or survival), the dependent variable. The resistance index (RI) was calculated as the ratio of the GR_{50} or LD_{50} values between the GUI-R and A17-S biotypes.

Differences in GR_{50} and LD_{50} values between the GUI-R and A17-S populations were evaluated using the "Compare models" function in GraphPad Prism (version 10.5.0, Boston, USA, www.graphpad.com), which applies the extra-sum-of-squares F test to determine whether dose–response curves differ significantly in their inflection points. The analysis was performed using individual values from all replicates per dose and population, for each assay according to the MoA. Statistical significance was defined as $p < 0.05$. Normality was checked using the Shapiro-Wilk test, and homogeneity of variance was confirmed with the Spearman rank correlation. For the Shikimate and EPSPS genomic copy number assays, $p < 0.05$ from ANOVA was considered significant, and means were separated using the Tukey HSD test ($\alpha = 0.05$). Significant differences were denoted by different letters.

3. Results

3.1. Dose-response assays

The susceptible A17-S population was totally controlled by all MoAs at rates equal to or below the field rate (i.e., 800 g ae ha^{-1} for glyphosate, 7.5 g ai ha^{-1} for thifensulfuron, and 37.5 g ae ha^{-1} for 2,4-D and MCPA).

The GUI-R population showed low levels of resistance to the EPSPS inhibitor glyphosate (Fig. 1A-B). Based on GR_{50} or LD_{50} RI, it was more than twice as resistant compared to the A17-S population (Table 2). Moreover, the GR_{90} and LD_{90} values for GUI-R were 1303 g ae ha^{-1} and 1235 g ae ha^{-1}, respectively, both of which were well above the field rate confirming resistance evolution.

GUI-R population showed high levels of resistance to the ALS-SU inhibitor thifensulfuron (Fig. 1C-D). The GR_{50} RI was 340.6 and LD_{50} RI was 123 more resistant than compared to the GR_{50} and LD_{50} values of

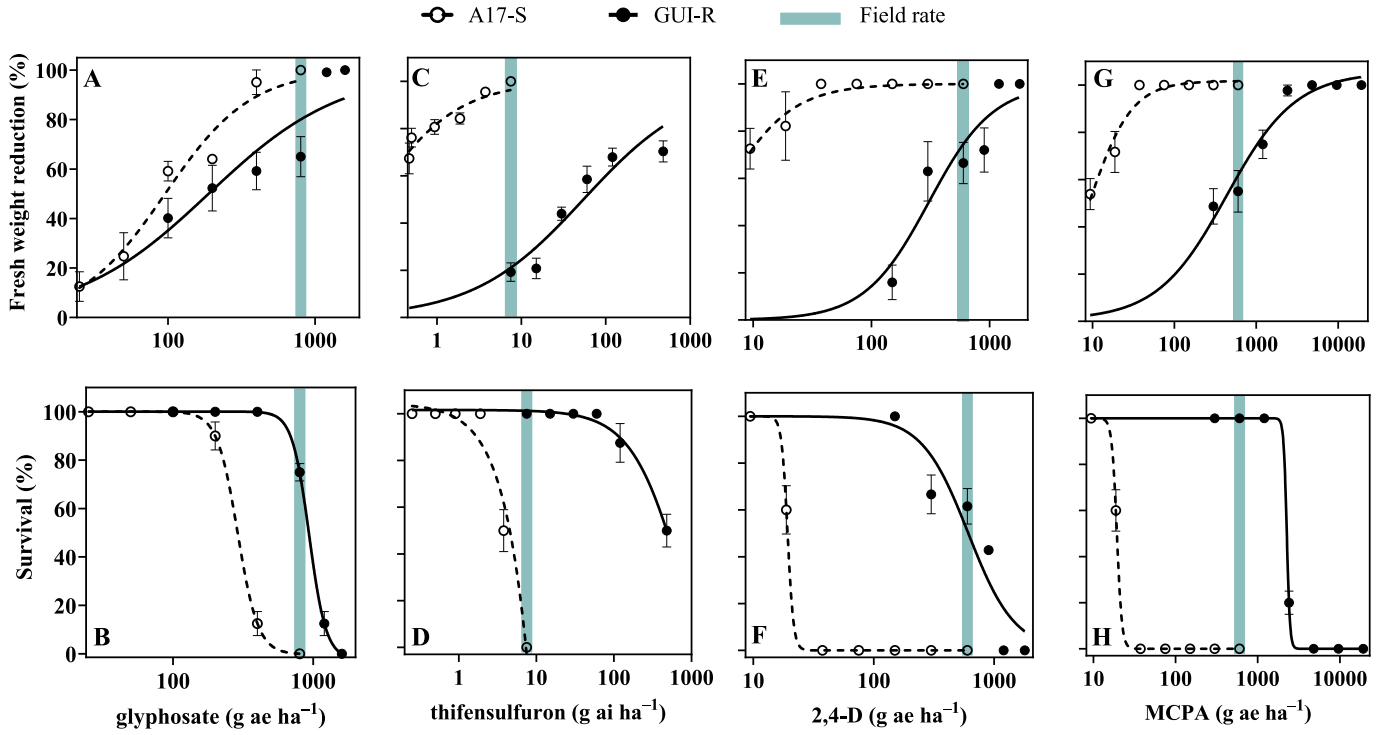

Fig. 1. Dose-response curves for fresh weight reduction and survival in *Bassia scoparia* populations, resistant (GUI-R) and susceptible (A17-S) to glyphosate (A–B), thifensulfuron (C—D), 2,4-D (E–F), and MCPA (G–H). Vertical bars represent standard errors.

Table 2
Estimated parameters from nonlinear regression models for fresh weight reduction and survival in *Bassia scoparia* populations treated with three herbicide modes of action (MoAs).[a]

Herbicides	Population	Fresh weight reduction				Survival			
		Slope	GR_{50}	RI_{50}	GR_{90}	Slope	LD_{50}	RI_{50}	LD_{90}
Glyphosate	A17-S	1.5 ± 0.20	95.3 ± 9.3	–	412.3	−5.98 ± 0.02	288 ± 0.44	–	416
	GUI-R	0.96 ± 0.28	188.8 ± 53*	2	1303	−7.6 ± 0.18	925 ± 4.1*	3.2	1235
Thifensulfuron	A17-S	0.86 ± 0.16	0.17 ± 0.05	–	2.9	−3.82 ± 0.59	3.9 ± 0.14	–	6.9
	GUI-R	0.66 ± 0.13	54.5 ± 11*	340.6	1521	−20.63 ± 0.14	480 ± 4.7 *	123	534
2,4-D	A17-S	1.49 ± 0.32	5.13 ± 1	–	22.4	−20.77 ± 0.26	19.1 ± 0.05	–	21.2
	GUI-R	1.59 ± 0.42	306.8 ± 56*	59.8	1195	−2.25 ± 0.85	610.8 ± 112*	32	1622
MCPA	A17-S	1.79 ± 0.24	9.1 ± 0.65	–	31	−14.13 ± 0.22	19.3 ± 0.01	–	22.5
	GUI-R	1.19 ± 0.22	389 ± 56*	42.7	2465	−19.3 ± 11	1117 ± 48*	57.9	1251

[a] $GR_{50/90}$ and $LD_{50/90}$ represent the effective doses (expressed in g ai/ae ha^{-1}) required to achieve 50 % and 90 % reductions in plant biomass and survival, respectively. $RI_{50/90}$ refers to the Resistance Index, which represents the ratio between the parameters ($GR_{50/90}$ and $LD_{50/90}$) of the R and S populations. The *p*-value (probability level of significance of the nonlinear model) was <0.0001. Asterisks indicate significant differences between the S and R populations in response to herbicide treatment ($p < 0.05$). ± denotes the standard error.

the A17-S population, 0.17 g ai ha^{-1} and 3.9 g ai ha^{-1}, respectively (Table 2). The high resistance in GUI-R was further confirmed by their GR_{90} (1521 g ai ha^{-1}) or LD_{90} (534 g ai ha^{-1}) values.

GUI-R population displayed characteristic damage symptoms, such as epinasty and reduced growth when treated with synthetic auxin herbicides. However, it dramatically recovered one week after treatment with 2,4-D or MCPA (Supplementary material. Spreadsheet 6). Consequently, high resistance to both synthetic auxin herbicides was observed in the GUI-R population (Fig. 1E-F and G-H), with GR_{50} RI greater than 40 times and LD_{50} RI greater than 30 times compared to the A17-S population (Table 2). The GR_{90} and LD_{90} values followed a similar pattern.

3.2. Cross-resistance to herbicides and 2,4-D metabolism

The GUI-R population did not exhibit cross-resistance to dicamba or fluroxypyr, showing control levels of 76 % (144 g ae ha^{-1}) and 100 % (100 g ae ha^{-1}), respectively, at the lowest dose (Supplementary material. Spreadsheet 2). In contrast, a single-dose imazamox test revealed cross-resistance to ALS-IMI in the GUI-R population, with 46 % of plants surviving the label rate (data not shown). Interestingly, malathion pre-treatment reversed GUI-R resistance to 2,4-D and reduced biomass by 97 % compared to the control treated only with 2,4-D (Supplementary material. Spreadsheet 3).

3.3. Shikimate assay and EPSPS molecular characterization

Shikimate content in untreated plants of the GUI-R and A17-S populations was low, with concentrations of 0.47 and 0.29 μg mL^{-1}, respectively. Following glyphosate treatment, shikimate accumulation in the A17-S population increased substantially (0.91 μg mL^{-1}), reaching levels up to 3.1-fold higher than those observed in the untreated control. In contrast, shikimate levels in the GUI-R population remained relatively stable, with a maximum concentration of 0.59 μg mL^{-1}, indicating a 1.3-fold increase compared to the control (Fig. 2). Sequencing of the *EPSPS* gene in the GUI-R and A17-S populations did not reveal nucleotide substitutions at positions Thr102, Ala103, and Pro106 (Supplementary material. Spreadsheet 4). However, the average *EPSPS:ALS* gene copy number in GUI-R (4.9 copies) was significantly higher than that in the A17-S population (0.59 copies). Additionally, the GUI-R population showed high intra-population variability, ranging from 2.38 to 8.69 copies. In contrast, the A17-S population exhibited a lower number of *EPSPS:ALS* gene copies, ranging from 0.45 to 1.01, with limited variability (Fig. 3).

3.4. ALS gene sequencing

In the A17-S population, no substitutions were found at any of the positions in the *ALS* gene associated with resistance to ALS inhibitors. In contrast, all individuals from the GUI-R population exhibited different amino acid substitutions (Supplementary material. Spreadsheet 4). Six plants had Pro197Ser (four homozygous and two heterozygous), four plants had Pro197Leu (all homozygous), and two plants had heterozygous Trp574Leu, either alone or combined with Pro197Ser (Table 3). Chromatogram analysis revealed that the overall mutations were 67 % ($n = 8$) homozygous and 33 % ($n = 4$) heterozygous (overlapping peaks).

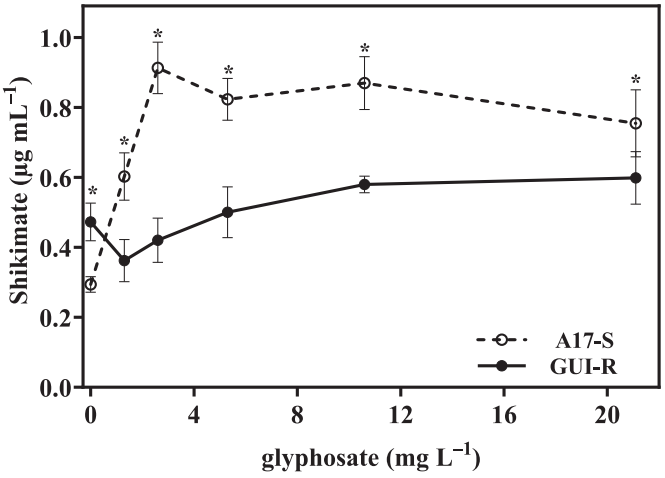

Fig. 2. Accumulated shikimate in sensitive (A17-S, dashed line) and resistant (GUI-R, continuous line) *Bassia scoparia* populations at increasing glyphosate concentrations. The vertical bars represent the standard errors. Asterisks above the bars indicate statistically significant differences ($p < 0.05$).

4. Discussion

This study confirms, for the first time in Europe, triple resistance to EPSPS, ALS, and synthetic auxin MoA in a *B. scoparia* population from no-till winter cereals, under a conventional management system heavily dependent on herbicide use. The selection pressure imposed by different herbicide MoAs at various crop stages highlights the rapid spread (mediated by pollen and seed) and persistence of resistance genes within and across field populations (Beckie et al., 2016).

The evolution of multiple resistance is common in *B. scoparia*. For instance, populations with triple resistance to ALS, EPSPS, and synthetic auxins (dicamba/fluroxypyr) have been previously reported in Alberta, Canada (Beckie et al., 2019b). These populations have undergone rapid evolution, increasing in frequency from 10 % in 2017 to 45 % in 2021. Additionally, the coexistence of field and ruderal populations with different combinations of MoAs resistance (HRAC 2/9 and HRAC 2/4)

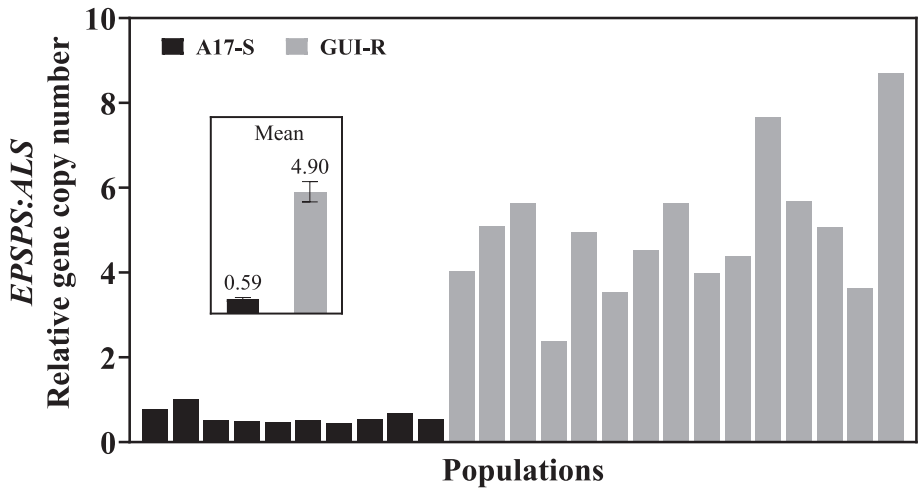

Fig. 3. Relative *EPSPS* gene copy number, normalized to the reference gene *ALS*, in two *Bassia scoparia* populations: A17-S (black bars) and GUI-R (gray bars). Each bar represents an individual plant. The box in the graph represents the mean, and the vertical bars indicate the standard error.

Table 3
Amino acid substitutions detected in the *ALS* gene of the GUI-R *Bassia scoparia* population.

Sustitutions[a]	Plants	Frequency (%)[b]	Homozygosity[c]	Heterozygosity[c]
Pro197Ser CCG → **T**CG	6	50	4 (33.3)	2 (16.6)
Pro197Leu CCG → **C**TG	4	33.3	4 (33.3)	
Trp574Leu TGG → **T**TG	1	8.3		1 (8.3)
Pro197Ser + Trp574Leu	1	8.3		1 (8.3)

[a] Codon position and nucleotide change (bold) corresponding to the amino acid substitution.
[b] Frequency (%) of each amino acid substitution relative to the total number of plants analyzed.
[c] Individuals with each amino acid substitution (homozygous or heterozygous); frequency (%) in parentheses.

was observed, suggesting that this resistance has not been exclusively selected in agricultural fields (Beckie et al., 2019a; Geddes et al., 2022, 2023). In addition, a *B. scoparia* population from croplands in Kansas, USA, was also found to be resistant to four herbicide MoAs: PSII, ALS, and EPSPS inhibitors, as well as synthetic auxins (dicamba). The TSR mechanisms conferring resistance to atrazine, chlorsulfuron, and glyphosate were identified as the Ser264Gly mutation in the *psbA* gene, Pro197Thr and Trp574Leu mutations in the *ALS* gene, and an increased *EPSPS* gene copy number (Varanasi et al., 2015).

4.1. Glyphosate resistance

In this study, the GUI-R population was found to be 2- to 3.2-fold more resistant to glyphosate based on RI_{50} values for biomass reduction and survival. Although the resistance level, caused by increased *EPSPS* gene copy number, is considered low, the estimated GR_{90} and LD_{90} values indicate an evolving higher level of glyphosate resistance, and the field dose is insufficient for effective population control.

Previous studies have also shown that glyphosate resistance in *B. scoparia* was associated with an increased *EPSPS* gene copy number, typically ranging from 3 to 11 copies, arranged in tandem, allowing the production of more EPSPS enzymes than glyphosate can inhibit (Jugulam et al., 2014; Patterson et al., 2018, 2019; Wiersma et al., 2015). Furthermore, resistance levels in glyphosate-resistant *B. scoparia* populations increased with the number of *EPSPS* gene copies (Gaines et al., 2016; Kumar et al., 2018; Kumar and Jha, 2015). Some glyphosate-resistant *B. scoparia* populations with two to four *EPSPS* gene copies have been shown to survive the field dose but were effectively controlled with a five-fold higher dose. In contrast, others with five to fourteen *EPSPS* gene copies were able to survive doses up to five times the field rate (Kumar and Jha, 2015; Lim et al., 2021).

The low level of resistance observed in GUI-R population suggests that it is in early stages of glyphosate resistance evolution. However, the wide range of *EPSPS* gene copy numbers indicates high intra-population genetic variability, leading to differential control levels. This suggests that sequential glyphosate applications and the absence of a fitness cost associated with resistance (Lim et al., 2021; Osipitan and Dille, 2019) will likely favor the selection and persistence of individuals with higher *EPSPS* gene copy numbers in the field.

4.2. ALS inhibitors resistance

In this study, *ALS* gene sequencing of the GUI-R population revealed different substitutions and zygosity statuses at 197 and 574 mutation sites, including homozygous and heterozygous Pro197Ser, homozygous Pro197Leu, heterozygous Trp574Leu and a combination of heterozygous Trp574Leu + Pro197Ser. Consequently, GUI-R exhibited 340-fold and 480-fold resistance to thifensulfuron, based on fresh weight reduction and survival, respectively. Mutations at Pro197 are typically SU-specific, whereas Trp574 confers broad cross-resistance to both SU and IMI herbicides (Yu and Powles, 2014). In *B. scoparia*, common Pro197 substitutions involve Thr, Leu, Ser, Ala, Arg, or Gln, while Trp574 is often substituted by Leu or Arg (Légère et al., 2013; Tranel et al., 2025). These mutations confer extreme resistance in *B. scoparia* as also observed in this study, with levels ranging from 30-fold to over 500-fold, and in some cases, up to 28,000-fold (Foes et al., 1999; Kumar et al., 2015; Warwick et al., 2008).

GUI-R also showed a degree of cross-resistance to imazamox, with 46 % of plants surviving the field dose (data not shown). Although IMI herbicides were not used in the sampled field, they were applied in adjacent fields (as detailed in supplementary material. Spreadsheet 5). Given the diversity of ALS mutations and the high potential for gene flow via, cross-pollination (Beckie et al., 2011; Warwick et al., 2008), this cross-resistance is not unexpected. The presence of *ALS* mutations in the plants of GUI-R population, along with *EPSPS* gene overexpression, further supports this hypothesis.

4.3. Synthetic auxins resistance

The GUI-R population was 60- and 42.7-fold more resistant to 2,4-D and MCPA, respectively, based on GR_{50} values, requiring doses of 1195 and 2465 g ae ha^{-1} to achieve 90 % biomass reduction. A *B. scoparia* population from Nebraska exhibited 12-fold resistance to 2,4-D, requiring 2619 g ae ha^{-1} for 50 % biomass reduction, and was 38- and 13-fold more resistant to dicamba and fluroxypyr, respectively (LeClere et al., 2018). The striking difference in resistance levels between these resistant biotypes was dependent on the susceptibility of the reference population. The A17-S population used in this study was highly susceptible, which magnified the resistance levels in the GUI-R population. Previous studies have shown that various inbred *B. scoparia* lines exhibit control rates ranging from 49 % to 75 % at the field dose of 2,4-D, requiring 550–1030 g ae ha^{-1} to cause visible injury in 50 % of the plants (Nandula and Manthey, 2002).

Among synthetic auxin-resistant *B. scoparia* populations, dicamba resistance is the most frequently reported (Heap, 2025) and is often linked to cross-resistance with fluroxypyr and 2,4-D (Dhanda et al., 2025; Geddes et al., 2022; Jha et al., 2015; Kumar et al., 2019b; LeClere et al., 2018). Recently, a transposable element (TE) was identified within a dicamba target-site gene, which alters splicing and reduces the perception of both synthetic and natural auxins, resulting in resistance to both dicamba and 2,4-D (Montgomery et al., 2024). However, such cross-resistance patterns were not observed in this study, as the GUI-R populations remained susceptible to both dicamba and fluroxypyr. This unique cross-resistance pattern suggests that the presence of TE-based TSR mechanism in the resistant population is unlikely. Nonetheless, further studies are needed to better understand the molecular underpinnings of this population.

NTSR mechanisms against synthetic auxins in *B. scoparia* have been associated with reduced translocation and enhanced metabolism (Ou et al., 2018; Pettinga et al., 2018; Todd et al., 2024). Enhanced metabolism of 2,4-D, primarily mediated by Phase I cytochrome P450 enzymes and Phase II glycosyltransferases (GT) and glutathione-S-transferases (GST), has been documented in multiple species (Palma-Bautista et al., 2020; Todd et al., 2020; Torra et al., 2024). In *B. scoparia*, the involvement of glucosyltransferases (GTs) and glutathione S-transferases (GSTs) has been reported in fluroxypyr-resistant populations (Todd et al., 2024). Although 2,4-D metabolism has not been previously reported as an exclusive resistance mechanism in this species, our preliminary results showed that malathion acted synergistically with 2,4-D at the field rate, reversing resistance in the GUI-R population. This suggests the potential involvement of cytochrome P450 enzymes in 2,4-D detoxification. Additional metabolic studies are needed to identify the metabolites involved and to assess other inhibitors to confirm the specific enzyme type and phase associated with metabolic resistance.

5. Conclusion

The assessment of herbicide resistance in a *B. scoparia* population (GUI-R) collected from a no-till winter cereal field in Catalonia, Spain, revealed triple resistance to EPSPS, ALS, and synthetic auxin herbicides. An increased *EPSPS* gene copy number was identified as the mechanism conferring glyphosate resistance. Different amino acid substitutions were detected in the *ALS* gene at positions Pro197 and/or Trp574, which conferred cross-resistance to thifensulfuron and imazamox. This population exhibited resistance to 2,4-D and MCPA but showed no cross-resistance to dicamba or fluroxypyr. This resistance was reversed when malathion, a cytochrome P450 inhibitor, was co-applied with 2,4-D at the label rate, suggesting that enhanced metabolism may underlie the observed resistance. Multiple resistance in *B. scoparia* reflects its high genetic variability and substantial gene flow, representing a major challenge for weed management, given its capacity to evolve and stack resistance mechanisms across different herbicide MoAs. These findings highlight the urgent need for integrated weed management strategies that combine cultural and mechanical practices with judicious chemical use to reduce selection pressure and limit the spread of resistant biotypes, which could negatively impact agricultural production.

CRediT authorship contribution statement

Germán Mora: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Miriam Gil-Monreal:** Writing – review & editing, Investigation, Supervision. **Maria Dolores Osuna:** Writing – review & editing, Methodology, Investigation, Supervision. **Vijaya Bhaskar Alwarnaidu Vijayarajan:** Writing – review & editing, Methodology, Investigation. **José María Montull:** Writing – review & editing, Methodology, Conceptualization. **Josep María Llenes:** Writing – review & editing, Methodology, Conceptualization. **Jordi Recasens:** Writing – review & editing, Project administration. **Alicia Cirujeda:** Writing – review & editing, Methodology. **Ana Isabel Marí:** Writing – review & editing, Methodology. **Joel Torra:** Writing – review & editing, Methodology, Supervision, Resources, Conceptualization, Funding acquisition.

Declaration of competing interest

The authors declare no conflicts of interest associated with this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2025.106529.

Data availability

Data will be made available on request.

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