Confirmation and characterisation of ALS inhibitor resistant Poa trivialis from Ireland

Pesticide Biochemistry and Physiology

Vijayarajan, Vijaya Bhaskar Alwarnaidu; Torra, Joel; Runge, Fabian; de Jong, Hans; van de Belt, José et al

https://doi.org/10.1016/j.pestbp.2024.106266

Pesticide Biochemistry and Physiology 208 (2025) 106266

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Confirmation and characterisation of ALS inhibitor resistant *Poa trivialis* from Ireland

Vijaya Bhaskar Alwarnaidu Vijayarajan [a,*], Joel Torra [b], Fabian Runge [c], Hans de Jong [d], José van de Belt [d], Michael Hennessy [a], Patrick Dermot Forristal [a]

[a] Crop Science Department, Teagasc Oak Park Research Centre, Carlow, Ireland
[b] Department of Agricultural and Forest Sciences and Engineering, University of Lleida – Agrotecnio CERCA Centre, Lleida, Spain
[c] IDENTXX GmbH, Stuttgart, Germany
[d] Laboratory of Genetics, Wageningen University & Research, Wageningen, Netherlands

ARTICLE INFO

Keywords:
Acetolactate synthase (ALS) inhibitors
Poa trivialis
Diploid
Target-site resistance (TSR)

ABSTRACT

Relying on one broad-spectrum product to control grass weeds has resulted in cases of resistance to acetolactate synthase (ALS) inhibitors in species that were not the primary target such as *Poa trivialis* (POATR), in wheat fields in Ireland. In this study, we have characterised ALS inhibitor resistance in two populations of POATR-R, suspected of being resistant, to (i) sulfonylurea (SUs)-mesosulfuron + iodosulfuron (the selecting agent), (ii) SU + sulfonylamino-carbonyl-triazolinone (SCT)-mesosulfuron + propoxycarbazone, and (iii) triazolopyrimidine (TP)-pyroxsulam ALS chemistries. Resistant POATR-R populations showed ALS inhibitor cross-resistance associated with target-site resistance (TSR) mutations. Combined mutations (Pro-197 and Trp-574) were found in POATR-R plants; Trp-574-Leu in POATR-R1 conferring greater SUs, SU + SCT and TP (resistance index, RI >214) resistance, while Pro-197-Thr in POATR-R2 (RI >37) was associated with less resistance. The high levels of ALS inhibitor cross-resistance in POATR-R populations caused by TSR mutations are consistent with the ploidy status, as cytogenetic tests revealed that both the R and S populations were diploid (2n = 2× = 14). Cytochrome P450 inhibitor assays did not detect metabolism-based ALS resistance in POATR-R. Alternative herbicide modes of action, including acetyl-CoA carboxylase (pinoxaden, fenoxaprop, propaquizafop, cycloxydim or clethodim) and 5-enolpyruvylshikimate-3-phosphate synthase (glyphosate) inhibitors applied at the recommended label rate were highly effective on these resistant populations. Residual herbicides and cultural methods, as well as the judicious use of alternative in-crop herbicide options, should be prioritized as sustainable options for managing these ALS-resistant populations. This is first worldwide characterisation of resistance mechanisms in *P. trivialis* to ALS inhibitor chemistries.

1. Introduction

The meadow grasses, of which *Poa annua* L. (annual bluegrass or annual meadow grass) and *Poa trivialis* L. (roughstalk bluegrass or roughstalk meadow grass) are the most common (BSBI, 2024), have been ranked as low priority in arable crops in Ireland as they have been easy to control (Vijayarajan et al., 2022). *Poa trivialis* usually infests field margins but can also ingress into crop fields (Marshall, 1985). Its growth is facilitated by the mild damp Atlantic climate and the change to a continuous cropping system from a previous mix of livestock and crop rotation (Alwarnaidu Vijayarajan et al., 2023). In crop fields, *P. trivialis* is wind-pollinated and can be diploid (2n = 2× = 14), triploid (2n = 3× = 21) or tetraploid (2n = 4× = 28) (Zonneveld, 2019). While it is mostly perennial, it often behaves as an annual due to weakly-developed stolons (Marshall, 1985; Brunharo et al., 2024). In the UK, which has a climate broadly similar to Ireland, the economic threshold for *P. trivialis* in winter cereals is 15 plants m^{-2}, suggesting that their ingress into crop fields, even with low plant populations may reduce cereal yields (Harvey, 1985).

In Ireland, spring barley (120,000 ha), winter wheat (60,000 ha) and winter barley (60,000 ha) are the dominant cereal crops (CSO, 2022). For winter cereals, wetter autumns with monthly rainfall ranging from 78.6 to 126.2 mm, fewer pre-emergence spraying opportunities, and the withdrawal of EU registration for isoproturon has resulted on increased

* Corresponding author at: Crop Research Centre Oak Park, Teagasc, Carlow R93 XE12, Ireland.
E-mail address: Vijaya.Bhaskar@teagasc.ie (V.B.A. Vijayarajan).

https://doi.org/10.1016/j.pestbp.2024.106266
Received 26 October 2024; Received in revised form 2 December 2024; Accepted 14 December 2024
Available online 20 December 2024

reliance on spring application of acetolactate synthase (ALS) inhibitors in winter wheat for grass weed control (Met Eireann, 2024; Alwarnaidu Vijayarajan et al., 2023). Among the ALS inhibitors, sulfonylureas (SUs, e.g., mesosulfuron, iodosulfuron), triazolopyrimidines (TPs, e.g., pyroxsulam) and sulfonylamino-carbonyl-triazolinones (SCTs, e.g., propoxycarbazone) are used to control *Bromus sterilis* L. (sterile brome or barren brome), which is the most widespread and competitive grass weed in Ireland, but these herbicides also control a range of other species in winter wheat (Vijayarajan et al., 2022). The ALS-inhibiting herbicides are commercially available only as formulated mixtures (Alwarnaidu Vijayarajan et al., 2023). The SUs-mesosulfuron + iodosulfuron is the most commonly used ALS chemistry by Irish growers due to its broad-range of grass-weed activity and cost-effectiveness (DAFM, 2016). This herbicide is the only one from ALS type registered in Ireland that claims control of *P. trivialis* in winter wheat. Repetitive use of broad-spectrum ALS inhibitors, as a sole weed control strategy, greatly increases resistance evolution-risks in high priority and lower priority target species, including *P. trivialis* (Alwarnaidu Vijayarajan et al., 2023).

Worldwide, populations of 175 (68 grass and 107 broadleaf) weed species have been identified as resistant to ALS inhibitors (Heap, 2024). Target-site resistance (TSR) and non-target-site resistance (NTSR) mechanisms can be responsible for ALS inhibitor resistance in grass weeds (Délye et al., 2013). Mutations at nine codon positions in the *ALS* gene, causing TSR, have been found to date, with Pro-197 and Trp-574 being the most common (Fang et al., 2022; Murphy and Tranel, 2019). In the case of NTSR to ALS inhibitors, enhanced herbicide metabolism or increased detoxification mainly through cytochrome P450 monooxygenases or other enzymes activity is predominant (Powles and Yu, 2010). Cytochrome P450 synergists, malathion or piperonyl butoxide (PBO) pre-treatment assays are often used as proxies to detect metabolism-mediated NTSR (Torra et al., 2021; Gaines et al., 2020).

Weed control failures in *P. trivialis* are relatively rare; only one case of ALS-SUs-mesosulfuron + iodosulfuron-resistant *P. trivialis* in a wheat field has been documented globally, but the mechanisms remain uncharacterised (Heap, 2024). Resistance monitoring has only recently commenced in Ireland and the programme has confirmed ALS inhibitor resistance in suspected populations of high priority species, *Lolium multiflorum* L. (Italian ryegrass) or *Alopecurus myosuroides* L. (blackgrass) (Alwarnaidu Vijayarajan et al., 2021; Vijayarajan et al., 2022) and less priority *P. annua* L. (annual bluegrass or annual meadow grass) (Alwarnaidu Vijayarajan et al., 2023). In 2023, for the first time, reports of weed control failures with SUs were received in populations of two *P. trivialis* (referred to as POATR-R1 and POATR-R2), which raised concerns. Both these fields have a history of ALS inhibitor use specifically, SUs-mesosulfuron + iodosulfuron in the spring to mainly target *B. sterilis*. Both POATR-R populations survived the field application of SUs-mesosulfuron + iodosulfuron, which would normally be effective in controlling *P. trivialis* in winter wheat. Our preliminary glasshouse screening also confirmed that POATR-R1 and POATR-R2 were resistant to the recommended label rate of mesosulfuron + iodosulfuron. The objectives of this study were to: (i) determine the resistance and cross-resistance levels in POATR-R1 and POATR-R2 to ALS inhibitors chemistries: SUs-mesosulfuron + iodosulfuron, SU + SCT-mesosulfuron + propoxycarbazone and TP-pyroxsulam, (ii) investigate the mechanisms (TSR and NTSR) involved in ALS inhibitor resistance and determine ploidy status, and (iii) assess the efficacy of alternative herbicides for controlling ALS-resistant populations.

2. Materials and methods

2.1. Plant materials and growing conditions

Seed populations of POATR-R1 and POATR-R2 were both from the same region in Ireland (County Meath) but from separate fields (53°59′N–6°49′W and 53°73′N–6°54′W, respectively) located *c.* 15 km apart. Samples were taken by growers and sent for testing, with just a limited seed supply for the assays. A sensitive (S) POATR population was obtained from a commercial seed supplier (Fruit Hill Farm, County Cork, Ireland) for use as a sensitive reference. The dose-response, alternative herbicide and NTSR screening assays were carried out on seedlings grown in glasshouse conditions. For these, the R and S seed populations were sown in 110 × 110 × 110 mm (nominal 1000 mL capacity) pots filled with a standard soil mix containing 70 % loam, 20 % horticultural grit, 10 % peat (medium) and 2 g L^{-1} of Osmocote Mini™ (National Agrochemical Distributors Ltd., County Dublin, Ireland). The pots were watered as required throughout the experiment. All plants were grown in a glasshouse compartment with 18 °C/12 °C (day/night) temperature regime at a photoperiod of 16 h supplemented with artificial lighting to maintain a minimum light intensity of 250 μ mol quanta m^{-2} s^{-1}.

2.2. Dose-response to ALS inhibitors

At the two-to-four leaf stage, the R and S populations were treated with a range of doses of three ALS inhibitors (Table 1). The selected dose rates for the R populations ranged from 0.25-to-8 times the recommended label rate and for S population ranged from 0.0625-to-2 times the recommended label rate. The herbicide was applied using a Generation III Research Track Sprayer (DeVries Manufacturing, Hollandale, MN, USA) with a Teejet 8002-EVS flat fan nozzle, delivering an output of 200 L ha^{-1} at a pressure of 250 kPa and speed of 1.2 ms^{-1}. The experiment was randomized with four replicates, with at least six plants per population per replicate for each dose. At 30 days post-treatment, aboveground plant material was harvested from each replicate and weighed. The shoot biomass (fresh weight) for each replicate was expressed as the percentage of the mean shoot biomass of the non-treated controls.

2.3. Efficacy of alternative non-ALS herbicides

At the two-to-four leaf stage, the R and S populations were tested with the recommended label rate of five ACCase (acetyl-CoA carboxylase) inhibitors pinoxaden (Axial Pro®, Syngenta, Cambridge, UK) at 30.3 g ai ha^{-1}, fenoxaprop (Foxtrot EW® FMC Agro Ltd., Flintshire, UK) at 82.8 g ai ha^{-1}, propaquizafop (Falcon® Adama, Reading, UK) at 150 g ai ha^{-1}, cycloxydim (Stratos® Ultra, BASF, Stockport, UK) at 150 g ai ha^{-1} and clethodim (Centurion® Max, Arysta LifeScience, Nogueres, France) at 120 g ai ha^{-1} and one EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) inhibitor glyphosate (Roundup® Flex, Monsanto, Cambridge, UK) at 540 g ai ha^{-1}. Each treatment had three replicates of each population, with at least six plants per population, and the entire experiment was repeated. Percent plant survival was assessed visually at 30 days post-treatment.

2.4. Identification of TSR mutations

Leaf samples were taken from eight plants of the R and S populations 30 days post-treatment for TSR analyses, from the preliminary screening. The samples for R populations were collected from plants that had survived the recommended label rate of SUs-mesosulfuron + iodosulfuron and the samples for S population was from non-treated control plants.

The DNA isolation, amplification and sequencing was carried out by IDENTXX GmbH (Stuttgart, Germany). Air-dried leaf samples (~0.5 cm^2) from a total of 24 leaves were placed in tubes containing two steel beads of 4.5-mm diameter and homogenised in a shaker mill (TissueLyser II, Qiagen, Hilden, Germany). Afterwards, DNA was extracted using a customized kit (Perkin Elmer, Rodgau; Chemagic Plant400 Kit) and using KingFisher™ Flex Magnetic Particle Processor (Thermo Fisher Scientific, Schwerte, Germany). Polymerase chain reaction (PCR) was performed on the genomic DNA (5 to 10 ng μL^{-1}) using specific primer combinations (Table 2) for amplification of gene fragments covering the two most common mutation sites, i.e., positions Pro-197 and Trp-574 of

Table 1
Herbicides used for dose-response assays in sensitive and resistant *Poa trivialis* populations.

Mode of Action	Chemical families	Active ingredient (ai)[a]	Trade name	Source	Dose of component active ingredient (g ai ha^{-1})[b]
ALS	Sulfonlyureas (SUs)	Mesosulfuron + iodosulfuron	Pacifica® Plus	Bayer CropScience Ltd., Cambridge, UK	0.9 + 0.3, 1.9 + 0.6, 3.8 + 1.3, 7.5 + 2.5, **15 + 5**, 22.5 + 7.5, 30 + 10, 60 + 20, 120 + 40 and 0
	Sulfonylurea (SU) + sulfonylaminocarbonyl-triazolinone (SCT)	Mesosulfuron + propoxycarbazone	Monolith®	Bayer CropScience Ltd., Cambridge, UK	0.9 + 1.4, 1.9 + 2.8, 3.7 + 5.6, 7.4 + 11.1, **14.9 + 22.3**, 22.3 + 33.4, 29.7 + 44.6, 59.4 + 89.1, 118.8 + 178.2 and 0
	Triazolopyrimidine (TP)	Pyroxsulam	Broadway® Star	Corteva Agrisciences, Cambridge, UK	1.2, 2.4, 4.7, 9.4, **18.8**, 28.1, 37.5, 75.0, 150.1 and 0

[a] Treatments with mesosulfuron + iodosulfuron and mesosulfuron + propoxycarbazone were applied with 1 % *v*/v Biopower (alkylethersulfate sodium salt) adjuvant (Bayer CropScience Ltd., Cambridge, UK); treatments with pyroxsulam contained 1 % v/v Kantor (alkoxylated triglycerides) adjuvant (Interagro Ltd., Hertfordshire, UK).
[b] Figures in bold are the recommended label rate for each herbicide.

Table 2
Functioning sets of primers used to amplify partial acetolactate synthase (ALS) gene to detect target-site resistance (TSR) mutations in *Poa trivialis*.

Primer name	Primer sequence (5′- > 3′)	Product size (bp)	Targeted mutation site
Poa197-for Poa197-rev Poa197-seq	CRATGGTCGCCATCACGG CTTGGTGATGGAACGGGTGAC GTGCCGATCATGCGG	98	ALS Pro-197
Poa574-for Poa574-rev Poa574-seq	CCTCCCYGTTAAGGTGATGATACT GTAWGTGTGCGCCCGATTG CCTTGTAAAACCTGTCCT	97	ALS Trp-574

the *ALS* gene (Délye and Michel, 2005; Murphy and Tranel, 2019). The primer sets were designed by retrieving the partial *ALS* gene sequences of reference *P. annua* from the GenBank database (KM388810.1). Target DNA was amplified in a thermal cycler (T100 PCR thermal cycler, Bio-Rad Laboratories GmbH, Feldkirchen, Germany) using initial denaturation for 3 min at 95 °C, followed by 40 cycles consisting of 95 °C for 10 s, 60 °C (primer design was done as to enable the same annealing temperature for all used primer sets) for 35 s, 72 °C for 30 s and 72 °C for 5 min for final extension. Successful amplification was checked using agarose gel electrophoresis. The PCR products were analysed for single nucleotide polymorphisms (SNPs) using pyrosequencing on a PyroMark Q24 (Qiagen) using specific sequencing primers (Table 2) (Ronaghi and Elahi, 2002). During the sequencing reaction, all incorporated nucleotides of a short region covering the position of interest were detected and reported by creating a pyrogram in a pyrorun file. Subsequently, the file was read by the PyroMark Q24 software (v. 2.0.8) and visually evaluated for mutations.

2.5. Determination of chromosome number

Seeds of the R and S populations were germinated at 20 °C in petri-dishes with moistened filter for 8–10 days. Rootlets and leaf meristem were excised, pre-treated with 0.001 M 8-hydroxyquinoline for two and half hours at 20 °C and fixed in glacial acetic acid-96 % ethanol (1:3) for at least three hours, before being replaced by 70 % ethanol fixative, and stored at 4 °C overnight. Chromosome analyses and image processing followed the protocol in Kantama et al. (2017) and de Jong et al. (2023). Briefly, fixed materials were rinsed three times in water and 15 mM citrate buffer (pH 4.5) and were digested in 1 mL pectolytic enzyme solution (0.1 % cellulase, 0.1 % pectolyase and 0.1 % cytohelicase in citrate buffer) for 1 h. Meristems were then placed in a clean grease-free slide covered with acetic acid 45 %, and the tissues were cut with fine needles, under a dissecting microscope, to form a suspension of cells. After complete cell drying, cells were stained with DAPI/Vectashield, covered with a cover slip, and examined under a fluorescence microscope with 100×, NA 1.4 objective and filters for DAPI (40,6-Diamidino-2-phenylindole dihydrochloride) fluorescence. The images of selected cell complements were captured and examined with the image processing software of Adobe Photoshop 25.12 (https://www.adobe.com). Photoshop's count tool was used to estimate chromosome numbers.

2.6. NTSR assay with malathion and PBO

At the two-to-four leaf stage, the R and S populations were treated with 1000 g ha^{-1} malathion or 2222.2 g ha^{-1} PBO along with 0.1 % *v*/v Tween-80 (Merck Life Science Limited, County Wicklow, Ireland). Approximately three hours after pre-treatment with P450 inhibitors, different herbicide doses (0.125×, 0.25×, 1×, 2× and 4×) of SUs-mesosulfuron + iodosulfuron (x = 15 + 5 g ha^{-1}) or TP-pyroxsulam (x = 18.8 g ha^{-1}) were applied. As the response of POATR populations to SUs or SU + SCT was similar (**section 3.1**), the P450 inhibitor assays were only conducted with SUs and TP. The selected dose rates for R populations were 1×, 2× and 4× and for S populations were 0.125×, 0.25× and 1×. The experiment consisted of non-treated control, malathion-treated, PBO-treated, herbicide-treated, malathion + herbicide and PBO + herbicide. There were four replicates of each treatment, with at least six plants per population per replicate. At 30 days post-treatment, above-ground plant material was harvested from each replicate and shoot fresh weight recorded. The data was analysed using analysis of variance (ANOVA) and a post-hoc Tukey's pairwise test was used to separate the population mean for each herbicide rate.

2.7. Statistical analysis

R (v.3.6.3) was used to analyse the data. Dose-response models were fitted to the shoot fresh weight data using the *DRC* package and two models were chosen through the lack-of-fit *F*-tests ($P > 0.05$) (Ritz et al., 2015). A four-parameter log-logistic model to model the data of SUs-mesosulfuron + iodosulfuron and a three-parameter log-logistic model to model the data of SU + SCT-mesosulfuron + propoxycarbazone or TP-pyroxsulam. Fitted models estimated the growth rate GR_{50} (i.e., the effective dose rate required to obtain a growth reduction of 50 % relative to untreated plants). The resistance index (RI) was calculated as GR_{50} of R population divided by GR_{50} of sensitive reference.

3. Results

3.1. Dose-response to ALS inhibitors

The two POATR-R populations had different responses in growth to the different applied rates of herbicide as indicated by the different curves in Fig. 1 and the data, particularly resistance index, in Table 3. However, both populations exhibited similar cross resistance to the individual ALS inhibitor herbicides (Fig. 1 and **Table3**). The growth of

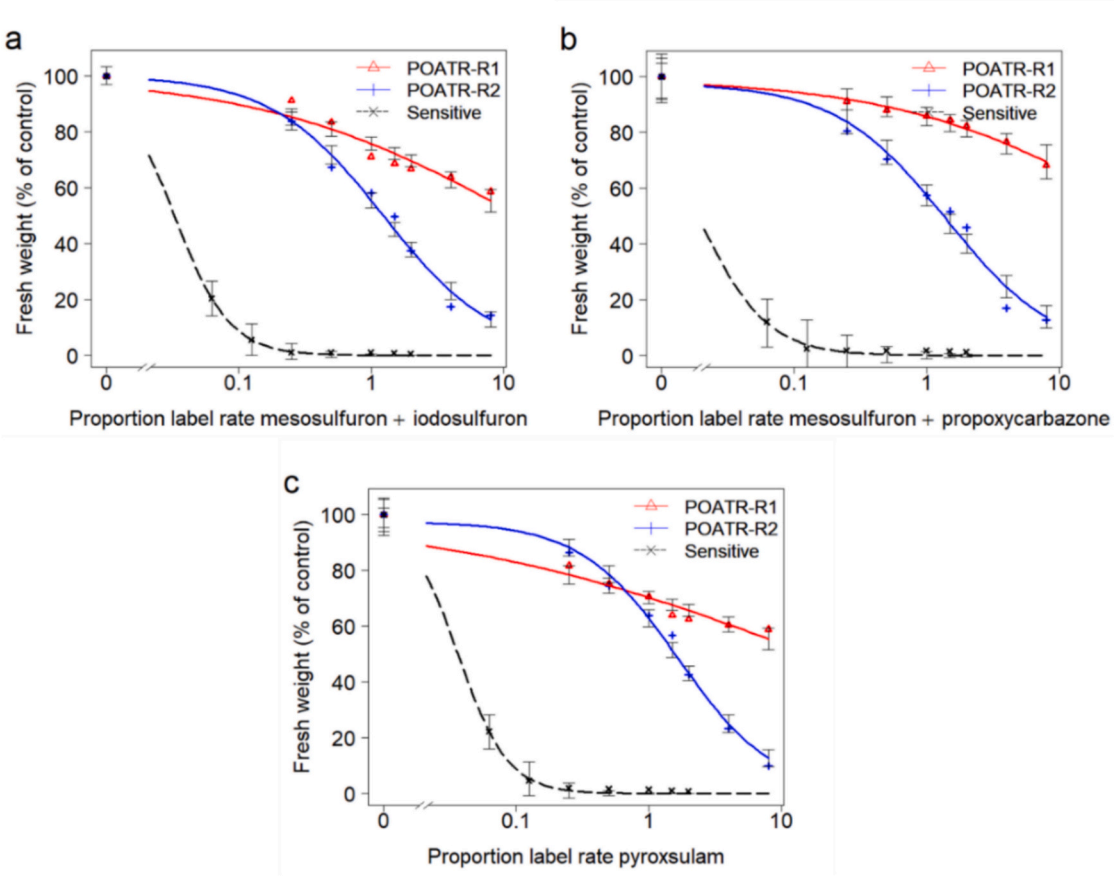

Fig. 1. Dose-response curves of sensitive and resistant populations of *Poa trivialis* treated with a range of rates expressed as proportion of the recommended label rate of ALS inhibitors SUs-mesosulfuron + iodosulfuron 15 + 5 g ai ha^{-1} (a), SU + SCT-mesosulfuron + propoxycarbazone 14.9 + 22.3 g ai ha^{-1} (b) and TP-pyroxsulam 18.8 g ai ha^{-1} (c) chemistries. Vertical bars represent the standard errors.

Table 3
Shoot fresh weight GR_{50} values (standard errors) of sensitive and resistant populations of *Poa trivialis* treated with a range of ± recommended label rates of ALS inhibitors SUs mesosulfuron + iodosulfuron 15 + 5 g ai ha^{-1}, SU + SCT mesosulfuron + propoxycarbazone 14.9 + 22.3 g ai ha^{-1} and TP pyroxsulam 18.8 g ai ha^{-1} chemistries. Resistance index (RI) was calculated as the ratio of GR_{50} values of R and S.

Population	Mesosulfuron + iodosulfuron		Mesosulfuron + propoxycarbazone		Pyroxsulam	
	GR_{50} (g ai ha^{-1})	RI	GR_{50} (g ai ha^{-1})	RI	GR_{50} (g ai ha^{-1})	RI
POATR-R1	>120 + >40	>240	>118.8 + >178.2	>237.6	>150.1	>214
POATR-R2	18.5 (1.15) + 6.2 (0.38)	37	21.0 (2.33) + 31.4 (3.48)	42	30.9 (2.17)	44
Sensitive	0.5 (0.14) + 0.2 (0.05)	–	0.5 (0.11) + 0.8 (0.16)	–	0.7 (0.17)	–

POATR-R1 did not fall below 50 %, even with the highest dose rate (8×), while the growth of POATR-R2 was impacted by herbicide dose with serious effects recorded at the higher rates, even though total control was not possible with the highest dose rate (8×). All GR_{50} values of POATR-R1 were estimated to exceed 8 times the recommended label rate, and for POATR-R2, they were well above the recommended label rate, with a high GR_{50} RI (RI >37). As expected, the S population was totally controlled by ALS inhibitors at rates equal to or below 0.5 times the recommended label rate, with biomass decreased by >90 %.

3.2. Efficacy of alternative non-ALS herbicides

Both R and S populations had similar responses ($P > 0.05$ for experiment x treatment interactions using ANOVA) to the recommended label rate of ACCase and EPSPS inhibitors, being sensitive (i.e. all treated plants died) to all herbicides tested, with a biomass reduction of >95 %.

3.3. Identification of TSR mutations

The R populations had two combined (Pro-197 and/or Trp-574) ALS mutations, compared to the S population (Table 4), suggesting that TSR is the dominant mechanism that confers a high cross-resistance in

Table 4
Amino acid (aa) substitutions identified at positions Pro-197 and Trp-574 in the *ALS* genes in *Poa trivialis* populations. Eight plants per population were analysed. Number of plants in which specific mutation(s) were detected are given in parenthesis. Mutant aa substitutions are in bold.

Population	Pro (CCG)-197		Trp (TGG)-574		Pro (CCG)-197 + Trp (TGG)-574	
	Codon	aa	Codon	aa	Codon	aa
POATR-R1	CCG (8)	Pro	TTG (7)	**Leu**	C/A-CG + T-G/T-G (1)	**Pro/Thr + Trp/Leu**
POATR-R2	ACG (3) + C/A-CG (1)	**Thr + Pro/Thr**	TTG (1) + T-G/T-G (1)	**Leu + Trp/Leu**	C/A-CG + T-G/T-G (2)	**Pro/Thr + Trp/Leu**
Sensitive	CCG (8)	Pro	TGG (8)	Trp		

POATR-R1 and POATR-R2 to ALS inhibitors chemistries (Table 3).

In POATR-R1, of the eight plants that were analysed, seven plants had homozygous Trp-574-Leu substitutions, and one plant had heterozygous Pro-197-Thr and Trp-574-Leu together. While in POATR-R2, four plants had Pro-197-Thr (three homozygous and one heterozygous), two plants had Trp-574-Leu (one each for homozygous and heterozygous) and two plants had heterozygous Pro-197-Thr and Trp-574-Leu together. Frequency of mutant positions and the level of zygosity have caused differences in resistance levels (Table 4); a high frequency of homozygous Trp-574-Leu in POATR-R1 gave RI >214; but in POATR-R2, combination of mutations (with a high frequency of Pro-197-Thr) and zygosity effects, conferred less resistance (RI >37) (Table 3).

3.4. Determination of chromosome number

Fourteen chromosomes were counted in both R and S populations, indicating that these populations were diploid (2n = 14) (Fig. 2). The diploid status of POATR-R1 and POATR-R2 is consistent with the phenotypic and genetic analysis, which demonstrated high levels of ALS cross-resistance endowed by TSR mutations.

3.5. NTSR assay with malathion and PBO

Applying either of the two P450 inhibitors in combination with herbicides (SUs-mesosulfuron + iodosulfuron or TP-pyroxsulam) did not cause significant change in the resulting biomass ($P > 0.05$) in POATR-R1 and POATR-R2 (**Supplementary I**). This implies that cytochrome P450-mediated NTSR is unlikely. The S population was totally controlled by SUs and TP at the recommended label rate, with or without the P450 synergists.

4. Discussion

The control of *P. trivialis* has not been a challenge in Irish crops to date, as it was achieved with low resistance-risk residual herbicides in conjunction with cultural methods, and this species was not considered high priority in weed control programmes (Vijayarajan et al., 2022). The loss of a key autumn post-emergence herbicide, isoproturon, coupled with a desire to control *B. sterilis* and other weeds in one spray application of a broad-spectrum product, has led to growers using much more SU mixes such as ALS-SUs-mesosulfuron + iodosulfuron (Alwarnaidu Vijayarajan et al., 2023). This single class approach allowed species that were not the primary target such as *P. trivialis* to be driven to resistance evolution.

In this study, we have confirmed and characterised the mechanisms of ALS inhibitor resistance in two populations of *P. trivialis* from winter wheat fields in Ireland. Both fields had a history of SUs-mesosulfuron + iodosulfuron use and growers had noticed ineffective weed control. Intensive use of SUs-mesosulfuron + iodosulfuron has selected a degree of cross-resistance to less commonly used SU + SCT-mesosulfuron + propoxycarbazone and TP-pyroxsulam (neither of which are labelled to control *P. trivialis*) in R populations (Table 3). Previous studies have also confirmed resistance to ALS inhibitors in two- or three-way herbicide formulations in other grass weeds (e.g., Davies et al., 2020; Collavo et al., 2012; Alwarnaidu Vijayarajan et al., 2023; Torra et al., 2021).

In resistant *P. trivialis*, combined substitutions Pro-197 and/or Trp-574 in POATR-R1 and POATR-R2, which were identified for the first time in this species, were associated with high levels of ALS cross-resistance (Table 4). The substitution Trp-574-Leu in POATR-R1, as also seen in many other ALS-resistant weeds (Tranel et al., 2024), caused greater resistance (RI >214) than in POATR-R2 (RI >37) with a high frequency of Pro-197-Thr. Co-existing substitutions Pro-197-Thr and Trp-574-Leu are quite common in cross-pollinated diploid species such as *Glebionis segetum* L. (corn marigold) (Papapanagiotou et al., 2023), *Sinapis alba* L. (white mustard) (Chtourou et al., 2024), *Apera spica-venti* L. (loose silky bent) (Košnarová et al., 2021), *Descurainia sophia* L. (flixweed) (Deng et al., 2017), and in several Irish populations of *A. myosuroides* and *L. multiflorum* (Alwarnaidu Vijayarajan et al., unpublished). The high levels of ALS cross-resistance caused by TSR mutations are consistent with the ploidy status (2n = 2× = 14) in R populations (Fig. 2), suggesting that resistance in *P. trivialis*, like that of most diploids species, is controlled by a single dominant or semi-dominant gene (Ghanizadeh et al., 2019; Ohta et al., 2024). Similar results were obtained in diploids such as *Amaranthus tuberculatus* L. (waterhemp) (Panozzo et al., 2013), *Lolium rigidum* L. (rigid ryegrass) and *A. myosuroides* (Yu et al., 2013), where TSR mutation in a single copy gene confers greater resistance to ALS or ACCase inhibitors; but in some higher plants, the same TSR mutation confers lesser resistance, which is likely due to polyploidy and gene dilution (Gaines et al., 2020).

Dual resistance mechanisms via TSR and metabolism-mediated NTSR to ALS inhibitors have been frequently reported in outcrossing diploid species such as *Lolium* spp. (ryegrass) (Torra et al., 2021; Scarabel et al., 2020), *Alopecurus* spp., (foxtail grasses) (Moss, 2017; Zhao et al., 2019), *Digitaria sanguinalis* (large crabgrass) (Zhao et al., 2023), *Papaver rhoeas* (corn poppy) (Rey-Caballero et al., 2017) or *Eurphorbia heterophylla* (wild poinsettia) (Rojano-Delgado et al., 2019). In this study, malathion or PBO did not confirm the presence of P450-mediated NTSR to SUs or TP in POATR-R (, suggesting the sole involvement of TSR in these populations, as confirmed by their high resistance index (POATR-R1: RI >214 and POATR-R2: RI >37).

In summary, we identified TSR in POATR-R for the first time in this species, which conferred cross-resistance to different classes of ALS inhibitors. This resistance has developed on farms relying on one broad-spectrum product or on one herbicide class for grass-weed control, which is not sustainable. Alternative non-ALS herbicides, ACCase-pinoxaden and fenoxaprop (used in wheat and barley) or ACCase-propaquizafop, cycloxydim and clethodim graminicides (used in oilseed rape) and EPSPS-glyphosate (used as pre-sowing burndown) were highly effective on POATR-R1 and POATR-R2. Most of the ACCase inhibitors except for fenoxaprop, are not labelled for control of *P. trivialis*

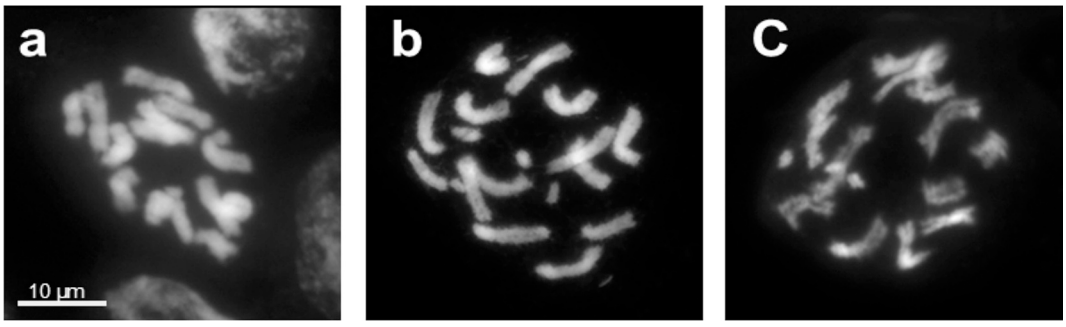

Fig. 2. DAPI image of representative cell complement from root tips of sensitive (a), POATR-R1 (b) and POATR-R2 (c) *Poa trivialis* populations, showing 14 chromosomes.

in Ireland. Spring application of ACCase inhibitors is an option in different crop types, but they are not a long-term tool for managing POATR-R, as they pose the same resistance-risk as ALS inhibitors (Moss et al., 2019), if used continuously as the sole control option. To manage ALS-resistant POATR-R populations multiple strategies, including the use of stacked residual herbicide modes of action, and cultural (e.g., the use of glyphosate as a part of stale seedbed, plough-based tillage, spraying-off distinct patches, spring cropping or inclusion of oilseed rape, establishing perennial grass-based mixtures at the field boundaries) tactics and strict machinery hygiene are essential. As Irish cropping systems is herbicide-dependent, resistance testing is crucial for identifying evolving problems and informing effective management options.

CRediT authorship contribution statement

Vijaya Bhaskar Alwarnaidu Vijayarajan: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Joel Torra:** Writing – review & editing, Methodology, Conceptualization. **Fabian Runge:** Writing – review & editing, Investigation. **Hans de Jong:** Writing – review & editing, Investigation. **José van de Belt:** Writing – review & editing, Investigation. **Michael Hennessy:** Writing – review & editing, Funding acquisition. **Patrick Dermot Forristal:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

Acknowledgements

This research was supported by funding from the project EVOLVE (Evolving grass-weed challenges and their impact on the adoption of carbon smart-tillage systems, Grant No: 2021R528) which is funded by the Department of Agriculture, Food, and the Marine (DAFM) under the Research Stimulus Fund (RSF) Programme. We are grateful to Gerard Nolan for glasshouse technical support. This work is a part of the Teagasc Climate Centre, which is a virtual centre that co-ordinates and disseminates agricultural climate change and biodiversity research and innovation in Ireland.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2024.106266.

References

Alwarnaidu Vijayarajan, V.B., Forristal, P.D., Cook, S.K., Schilder, D., Staples, J., Hennessy, M., Barth, S., 2021. First identification and characterization of cross- and multiple resistance to acetyl-CoA carboxylase (ACCase)- and acetolactate synthase (ALS)-inhibiting herbicides in black-grass (*Alopecurus myosuroides*) and Italian ryegrass (*Lolium multiflorum*) populations from Ireland. Agriculture 11 (12), 1272. https://doi.org/10.3390/agriculture11121272.

Alwarnaidu Vijayarajan, V.B., Morgan, C., Onkokesung, N., Cook, S.K., Hodkinson, T.R., Barth, S., Hennessy, M., Forristal, P.D., 2023. Characterization of mesosulfuron-methyl + iodosulfuron-methyl and pyroxsulam–resistant annual bluegrass (*Poa annua*) in an annual cropping system. Weed Sci. 71, 557–564. https://doi.org/10.1017/wsc.2023.55.

Brunharo, C.A.C.G., Benson, C.W., Huff, D.R., Lasky, J.R., 2024. Chromosome-scale genome assembly of *Poa trivialis* and population genomics reveal widespread gene flow in a cool-season grass seed production system. Plant Direct 8 (3), e575. https://doi.org/10.1002/pld3.575.

BSBI, 2024. Botanical Society of Britain and Ireland (BSBI). Durham, UK. https://bsbi.org/maps (accessed 13 August 2024).

Chtourou, M., Osuna, M.D., Vázquez-García, J.G., Lozano-Juste, J., De Prado, R., Torra, J., Souissi, T., 2024. Pro197Ser and the new Trp574Leu mutations together with enhanced metabolism contribute to cross-resistance to ALS inhibiting herbicides in *Sinapis alba*. Pestic. Biochem. Physiol. 201, 105882. https://doi.org/10.1016/j.pestbp.2024.105882.

Collavo, A., Strek, H., Beffa, R., Sattin, M., 2012. Management of an ACCase-inhibitor-resistant *Lolium rigidum* population based on the use of ALS inhibitors: weed population evolution observed over a 7 year field-scale investigation. Pest Manag. Sci. 69, 200–208. https://doi.org/10.1002/ps.3449.

CSO, 2022. Area, Yield and Production of Crops 2022. Central Statistics Office (CSO) County Cork, Ireland. https://www.cso.ie/en/releasesandpublications/ep/p-aypc/areayieldandproductionofcrops2022/data/ (accessed 13 August 2024).

DAFM, 2016. Pesticide usage in Ireland: Arable Crops Survey Report 2016. Pesticide Registration and Control Divisions (PRCD) of the Department of Agriculture, Food and the Marine (DAFM), County Kildare, Ireland (2016). https://www.pcs.agriculture.gov.ie/media/pesticides/content/sud/pesticidestatistics/ArableReport2016Final100620.pdf (accessed 17 August 2024).

Davies, L., Onkokesung, N., Brazier-Hicks, M., Edwards, R., Moss, S., 2020. Detection and characterization of resistance to acetolactate synthase inhibiting herbicides in *Anisantha* and *Bromus* species in the United Kingdom. Pest Manag. Sci. 76, 2473–2482. https://doi.org/10.1002/ps.5788.

Délye, C., Michel, S., 2005. "Universal" primers for PCR-sequencing of grass chloroplastic acetyl-CoA carboxylase domains involved in resistance to herbicides. Weed Res. 45, 323–330. https://doi.org/10.1111/j.1365-3180.2005.00467.x.

Délye, C., Jasieniuk, M., Le Corre, V., 2013. Deciphering the evolution of herbicide resistance in weeds. Trends Genet. 29 (11), 649–658.

Deng, W., Yang, Q., Zhang, Y., Jiao, H., Mei, Y., Li, X., Zheng, M., 2017. Cross-resistance patterns to acetolactate synthase (ALS)-inhibiting herbicides of flixweed (*Descurainia sophia* L.) conferred by different combinations of ALS isozymes with a Pro-197-Thr mutation or a novel Trp-574-Leu mutation. Pestic. Biochem. Physiol. 136, 41–45. https://doi.org/10.1016/j.pestbp.2016.08.006 0048-3575/.

Fang, J., Yang, D., Zhao, Z., Chen, J., Dong, L., 2022. A novel Phe-206-Leu mutation in acetolactate synthase confers resistance to penoxsulam in barnyard grass (*Echinochloa crus-galli* (L.) P. Beauv). Pest Manag. Sci. 78, 2560–2570. https://doi.org/10.1002/ps.6887.

Gaines, T.A., Duke, S.O., Morran, S., Rigon, C.A.G., Tranel, P.J., Küpper, A., Dayan, F.E., 2020. Mechanisms of evolved herbicide resistance. J. Biol. Chem. 295, 10307–10330. https://doi.org/10.1074/jbc.Rev120.013572.

Ghanizadeh, H., Buddenhagen, C.E., Harrington, K.C., James, T.K., 2019. The genetic inheritance of herbicide resistance in weeds. CRC Crit. Rev. Plant Sci. 38, 295–312. https://doi.org/10.1080/07352689.2019.1665769.

Harvey, J.J., 1985. Control of *Poa* spp. in winter cereals – is it worthwhile? Asp. Appl. Biol. 9, 117–128.

Heap, I.M., 2024. International Survey of Herbicide Resistant Weeds. http://www.weedscience.org (accessed 10 August 2024).

de Jong, H., van der Belt, J., Fransz, P., 2023. Critical steps in DAPI and FISH imaging of chromosome spread preparations. Methods Mol. Biol. 2672, 247–256.

Kantama, L., Erik Wijnker, E., de Jong, H., 2017. Optimization of cell spreading and imaging quality for the study of chromosomes in plant tissues. Methods Mol. Biol. 1669, 141–158.

Košnarová, P., Hamouz, P., Hamouzová, K., Linn, A., Sen, M.K, Mikulka, J., Šuk, J., Soukup, J., 2021. *Apera spica-venti* in the Czech Republic develops resistance to three herbicide modes of action. Weed Res 61, 420–429. https://doi.org/10.17221/427/2022-PSE.

Marshall, E.J.P., 1985. Field and field edge floras under different herbicide regimes at the Boxworth E. H. F. – Initial studies. In: Proceedings of the 1985 British Crop Protection Conference – Weeds; Nov 18–21. Brighton Metropole, UK, pp. 999–1006.

Met Eireann, 2024. The Irish Meteorological Service (Met Eireann), Dublin Ireland. https://www.met.ie/climate/available-data (accessed 13 August 2024).

Moss, S.R., 2017. Black-grass (*Alopecurus myosuroides*): why has this weed become such a problem in Western Europe and what are the solutions? Outlooks Pest Manag. 10, 207–212.

Moss, S.R., Ulber, L., den Hoed, I., 2019. A herbicide resistance risk matrix. Crop Prot. 115, 13–19. https://doi.org/10.1016/j.cropro.2018.09.005.

Murphy, B.P., Tranel, P.J., 2019. Target-Site mutations conferring herbicide resistance. Plants 8 (10), 382. https://doi.org/10.3390/plants8100382.

Ohta, K., Kawamata, E., Hori, T., Sada, T., 2024. Connecting genes to whole plants in dilution effect of target-site ALS inhibitor resistance of *Schoenoplectiella juncoides* (Roxb.) lye (Cyperaceae). Pestic. Biochem. Physiol. 203, 105984. https://doi.org/10.1016/j.pestbp.2024.105984.

Panozzo, S., Scarabel, L., Tranel, P.J., Sattin, M., 2013. Target-site resistance to ALS inhibitors in the polyploid species *Echinochloa crus-galli*. Pestic. Biochem. Physiol. 105, 93–101. https://doi.org/10.1016/j.pestbp.2012.12.003.

Papapanagiotou, A.P., Spanos, T., Zarrougui, N.E., Livieratos, I.C., Eleftherohorinos, I.G., 2023. Pro197 and Trp574 substitutions in the acetolactate synthase of corn marigold (*Glebionis segetum*) and their impact on competitive ability against barley. Weed Technol. 37, 165–173. https://doi.org/10.1017/wet.2023.17.

Powles, S.B., Yu, Q., 2010. Evolution in action: plants resistant to herbicides. Annu. Rev. Plant Biol. 61, 317–347. https://doi.org/10.1146/annurev-arplant-042809-112119.

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

Ritz, C., Baty, F., Streibig, J.C., Gerhard, D., 2015. Dose-response analysis using R. PLoS ONE 10, e0146021. https://doi.org/10.1371/journal.pone.0146021.

Rojano-Delgado, A.M., Portugal, J.M., Palma-Bautista, C., Alcántara-de la Cruz, R., Torra, J., Alcántara, E., De Pradoet, R., 2019. Target site as the main mechanism of resistance to imazamox in a *Euphorbia heterophylla* biotype. Sci. Rep. 9, 15423. https://doi.org/10.1038/s41598-019-51682-z.

Ronaghi, M., Elahi, E., 2002. Discovery of single nucleotide polymorphisms and mutations by pyrosequencing. Comp. Funct. Genom. 3, 51–56. https://doi.org/10.1002/cfg.132.

Scarabel, L., Panozzo, S., Loddo, D., Mathiassen, S., Kristensen, M., Kudsk, P., Gitsopoulous, T., Travlos, I., Tani, E., Chachalis, D., Sattin, M., 2020. Diversified resistance mechanisms in multi-resistant *Lolium spp*. in three European countries. Front. Plant Sci. 11, 608845. https://doi.org/10.3389/fpls.2020.608845.

Torra, J., Montull, J.M., Taberner, A., Onkokesung, N., Boonham, N., Edwards, R., 2021. Target-site and non-target-site resistance mechanisms confer multiple and cross-resistance to ALS and ACCase inhibiting herbicides in *Lolium rigidum* from Spain. Front. Plant Sci. 12, 625138 doi:10/3389/fpls.2021.625138.

Tranel, P.J., Wright, T.R., Heap, I.M., 2024. Mutations in Herbicide-resistant Weeds to Inhibition of Acetolactate Synthase. http://www.weedscience.com (accessed 21 August 2024).

Vijayarajan, V.B.A., Fealy, R.M., Cook, S.K., Onkokesung, N., Barth, S., Hennessy, M., Forristal, P.D., 2022. Grass-weed challenges, herbicide resistance status and weed control practices across crop establishment systems in Ireland's mild Atlantic climate. Front. Agron. 4, 1063773 doi:10/3389/fagro.2022.1063773.

Yu, Q., Ahmad-Hamdani, M.S., Han, H., Christoffers, M.J., Powles, S.B., 2013. Herbicide resistance-endowing ACCase gene mutations in hexaploid wild oat (*Avena fatua*): insights into resistance evolution in a hexaploid species. Heredity 110, 220–231. https://doi.org/10.1038/hdy.2012.69.

Zhao, B., Xu, X., Li, B., Qi, Z., Huang, J., Hu, A., Wang, G., Liu, X., 2023. Target-site mutation and enhanced metabolism endow resistance to nicosulfuron in a *Digitaria sanguinalis* population. Pestic. Biochem. Physiol. 194, 105488. https://doi.org/10.1016/j.pestbp.2023.105488.

Zhao, N., Yan, Y., Ge, L., Zhu, B., Liu, W., Wang, J., 2019. Target site mutations and cytochrome P450s confer resistance to fenoxaprop-P-ethyl and mesosulfuron-methyl in *Alopecurus aequalis*. Pest Manag. Sci. 75 (1), 204–214. https://doi.org/10.1002/ps.5089.

Zonneveld, B.J.M., 2019. The DNA weights per nucleus (genome size) of more than 2350 species of the Flora of the Netherlands, of which 1370 are new to science, including the pattern of their DNA peaks. Forum Geobotan. 8, 24–78.