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First Detection 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

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Abstract: Understanding the resistance spectrum and underlying genetic mechanisms is critical for managing herbicide-resistant populations. In this study, resistance to acetyl CoA carboxylase (ACCase) and acetolactate synthase (ALS) inhibitors was investigated in four suspected resistant populations of *Alopecurus myosuroides* (ALOMY-001 to ALOMY-004) and *Lolium multiflorum* (LOLMU-001 to LOLMU-004), collected from cereal production fields in Ireland. Glasshouse assays with three ALOMY-active herbicides [propaquizafop, cycloxydim (ACCase) and mesosulfuron + iodosulfuron (ALS)] or five LOLMU-active herbicides [pinoxaden, propaquizafop, cycloxydim (ACCase) and mesosulfuron + iodosulfuron, pyroxsulam (ALS)], and target-site resistance mechanism studies, based on pyrosequencing, were carried out in each of those populations. For *A. myosuroides*, Ile-1781-Leu ACCase mutation contributed to propaquizafop and cycloxydim resistance (shoot dry weight GR_{50} resistance factor (RF) = 7.5–35.5) in all ALOMY populations, and the independent Pro-197-Thr or Pro-197-Ser ALS mutation contributed to mesosulfuron + iodosulfuron resistance (RF = 3.6–6.6), in ALOMY-002 to ALOMY-004. Most of the analyzed plants for these mutations were homo/heterozygous combinations or only heterozygous. For *L. multiflorum*, phenotypic resistance to mesosulfuron + iodosulfuron (RF = 11.9–14.6) and pyroxsulam (RF = 2.3–3.1) was noted in all LOLMU populations, but the Pro-197-Gln or Pro-197-Leu ALS mutation (mostly in homozygous status) was identified in LOLMU-001, LOLMU-002 and LOLMU-004 only. Additionally, despite no known ACCase mutations in any LOLMU populations, LOLMU-002 survived pinoxaden and propaquizafop application (RF = 3.4 or 1.3), and LOLMU-003 survived pinoxaden (RF = 2.3), suggesting the possibility of non-target-site resistance mechanisms for ACCase and/or ALS resistance in these populations. Different resistance levels, as evidenced by a reduction in growth as dose increased above field rates in ALOMY and LOLMU, were due to variations in mutation rate and the level of heterozygosity, resulting in an overall resistance rating of low to moderate. This is the first study confirming cross- and multiple resistance to ACCase- and ALS-inhibiting herbicides, highlighting that resistance monitoring in *A. myosuroides* and *L. multiflorum* in Ireland is critical, and the adoption of integrated weed management strategies (chemical and non-chemical/cultural strategies) is essential.

Keywords: ACCase; ALS; *A. myosuroides*; *L. multiflorum*; herbicide resistance; target-site resistance

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1. Introduction

Black-grass (*Alopecurus myosuroides*) and Italian ryegrass (*Lolium multiflorum*) are the most problematic grass weeds threatening the sustainability of crop rotations dominated by autumn-sown cereals across north-western Europe [1]. They are particularly difficult to control in early-sown winter cereals established after non-inversion tillage, due to the high seed retention of freshly shed populations, and as peak seed germination between September and November coincides with the sowing and early development of winter cereals [2,3]. In the United Kingdom (UK), the economic threshold (i.e., weed density that causes a 5% yield loss) for *A. myosuroides* is just 12 plants m^{-2}, and for *L. multiflorum* it is 5 plants m^{-2}, suggesting that even low plant populations may reduce crop yields [4,5].

As *A. myosuroides* and *L. multiflorum* are genetically variable obligate outcrossers, with high fecundity and rapid seedbank turnover [6,7], they are at high-risk of evolving resistance from continuous herbicide use [8,9]. To date, *A. myosuroides* has evolved resistance to seven different herbicide modes of action (MOA) and *L. multiflorum* to eight different herbicide MOA, worldwide [10]. In the UK, *A. myosuroides* resistant to Herbicide Resistance Action Committee (HRAC) MOA groups 1 (legacy A) [acetyl-CoA carboxylase (ACCase) inhibitors], 2 (B) [acetolactate synthase (ALS) inhibitors], 3 (K1) (e.g., residual herbicide, pendimethalin) and 5 (C2) (e.g., isoproturon, which was banned in the European Union in 2017) has been reported [10]. Consequently, resistant *A. myosuroides* occurs on most UK crop farms that use herbicides as a single weed control method [11]. While herbicide-resistant *L. multiflorum* is not as widespread as resistant *A. myosuroides* in the UK or in Europe [1], cases are now increasing [12] and populations resistant to herbicide MOA groups 1, 2, 5, 9 (G) (e.g., non-selective herbicide, glyphosate) and 15 (K3) (e.g., residual herbicide, flufenacet) have been reported [10]. Importantly, more than 75% of resistance cases within the UK and Europe are associated with two MOA groups: ACCase/ALS inhibitors.

The ACCase inhibitors control grass weeds by inhibiting acetyl CoA carboxylase, the enzyme required for fatty acid biosynthesis, and include herbicide chemistries of phenylpyrazoline (DEN), aryloxyphenoxypropionate (FOP), and cyclohexanedione (DIM) [13]. The ALS inhibitors control grass weeds by inhibiting acetolactate synthase, a key enzyme leading to the pathway to the branched-chain amino acids valine, leucine and isoleucine, and include herbicide chemistries of sulfonylurea (SU), triazolopyrimidine (TP), imidazolinone, pyrimidinyl-thiobenzoate and sulfonyl-aminocarbonyl-triazolinone [14]. Resistance to ACCase- and ALS-inhibiting herbicides, based on, both target-site resistance (TSR) due to mutations in the target genes, and non-target-site resistance (NTSR) usually through enhanced metabolism, has been reported in field populations of *A. myosuroides* and *Lolium* spp. in various regions [11,12,15–18].

In Ireland, agriculture area accounts for 65% (approximately 4.5 million ha) of the total land area [19]. Of this agricultural area, approximately 92% is devoted to grassland and 8% to arable cereal and crop production. Crop production in Ireland has evolved over the last 50 years from a mixed enterprise farming base, where spring cereals were grown in rotation as a part of grassland system, to current specialized cereal monoculture or rotation with all annual crops [20], which has resulted in much less proliferation of *A. myosuroides*. While the first occurrence of *A. myosuroides* was reported in 1987 [21], it was only recorded as a problem in isolated cases, in contrast to the UK or parts of Europe. *Lolium multiflorum* can occur as a weed of cereal crops in mixed rotations, which include grass, however, it has not been reported as a serious weed in cereal rotations on farms in Ireland, until recently. Non-selective and post-emergent herbicides have been the mainstay for weed control in arable rotations [22]. Growers exclusively rely on post-emergent ACCase- and ALS-inhibiting herbicides, which are the only remaining MOA available for selective grass weed control within cereal and broad-leaved crops (with the exception of herbicide MOA group 3, propyzamide, which provides largely residual control of a range of grass weeds in winter oilseed rape) [23]. The main registered commercial ACCase- and ALS-inhibiting herbicides are targeted for the control of *Avena fatua* (wild oats) or *Anisantha sterilis* (sterile brome), which are, the most competitive grass weeds in cereal crops in Ireland. This use

may have inadvertently influenced potential *A. myosuroides* and *L. multiflorum* control and resistance evolution.

In Ireland, more than 139,000 ha of cereal, oilseed rape and bean crops were treated with ACCase- and ALS-inhibiting herbicides in 2016, with ACCase-DEN-pinoxaden being the most widely used herbicide to control *A. fatua*, on at least 114,927 ha of wheat and barley (spring and winter) area, which represents an 11% increase in pinoxaden-treated area, from 2012 [22]. The ACCase-FOP-propaquizafop and ACCase-DIM-cycloxydim were used on 7792 ha (3% decrease) of broad-leaved crops, including oilseed rape and beans to control *A. fatua* and *A. sterilis*, while ALS-SU-mesosulfuron + iodosulfuron was used on 13,664 ha of winter wheat (0.4% increase) and ALS-TP-pyroxsulam was used on 3367 ha (29% increase) of winter wheat. Many of these *A. fatua* herbicides are also active on *L multiflorum* while these herbicides other than the ACCase-pinoxaden and ALS-pyroxsulam are active on *A. myosuroides*. It is logical to expect that recent practices may have allowed some degree of resistance to develop in isolated *A. myosuroides* and *L. multiflorum* populations, to ACCase and/or ALS inhibitors, even though they may not have been the intended target.

Monitoring of herbicide-resistant grass weeds in Ireland's climate and cropping system is only recently being addressed unlike in the UK, as these challenges developed later [23]. A recent grass weed survey in Ireland found suspect *A. myosuroides* and *L. multiflorum* strains that were difficult to control during the growing season. To date, no reports of herbicide resistance have been reported for control failures of *A. myosuroides* and/or *L. multiflorum* in Ireland. The aims of the present study were to: (a) determine the resistance patterns and the resistance levels to ACCase- and ALS-inhibiting herbicides in the suspected resistant populations of *A. myosuroides* and *L. multiflorum*, collected from cereal production fields in Ireland, and (b) to verify whether a TSR mechanism is involved in order to identify the mutation(s) that confer resistance. This knowledge is critical to develop integrated weed management (IWM) strategies to eliminate or control these resistant populations at a critical point in the development of weed challenges in this region.

2. Materials and Methods

2.1. Seed Materials and Growing Conditions

A nationwide observational survey of 136 farms was conducted from June to September 2020, which included a questionnaire, grass weed assessments, and the collection of seed samples for glasshouse resistance testing (Vijaya Bhaskar et al. unpublished). The survey farms were selected using a stratified pattern, to ensure different types of crop establishment systems were represented, using grower lists provided by Teagasc (the state-funded research and advisory service) and commercial crop advisors. The study fields within farms were identified on the basis of specific crop establishment systems practiced. A total of 157 field samples (approx. one sample per farm) of the most problematic grass weed species specified by each grower: *A. fatua* (73 populations), *Anisantha* spp. and *Bromus* spp., (52 populations), *Phalaris minor* (12 populations), *A. myosuroides* (12 populations) and *L. multiflorum* (8 populations) were collected. From this survey, we found four *A. myosuroides* (ALOMY-001 to ALOMY-004) and four *L. multiflorum* (LOLMU-001 to LOLMU-004) populations, with a high herbicide survival rate in the fields, and growers had reported control failures or difficult-to-control with ACCase- and/or ALS-inhibiting herbicides at recommended field rate, that were used in this study.

Information regarding the position and field history of each of the suspected resistant populations is given in Tables 1 and 2. In addition, a known ACCase/ALS-susceptible *A. myosuroides* population S-006 (Table 1), and a susceptible *L. multiflorum* population (SORG-002) from an organic seed supplier (Fruit Hill Farm, Co. Cork, Ireland) were used as susceptible controls. The fields within Co. Meath for ALOMY-002 and ALOMY-003, and LOLMU-003 and LOLMU-004, were located *c.* 25.6 km and *c.* 31.2 km apart, respectively. Seeds from suspect resistant and susceptible populations were collected, air-dried and stored in paper bags at 15 °C.

Table 1. Location and field history of the suspected resistant (ALOMY-001 to ALOMY-004) and susceptible (S-006) populations of *A. myosuroides* collected from cereal production fields prior to 2020-harvest for resistance testing.

Population	County	Field Position	Year	Establishment System	Crop	ALOMY-Active Herbicides Applied
ALOMY-001	Cork	51°71′ N–8°65′ W	2016–2020	Plough	Winter barley	None
ALOMY-002	Meath	53°48′ N–6°41′ W	2020	Plough	Spring barley	None
			2019	Plough	Winter wheat	
			2018	Plough	Winter wheat	
			2017	Plough	Winter oats	
			2016	Plough	Winter wheat	
ALOMY-003	Meath	53°56′ N–6°58′ W	2016–2020	Plough	Winter wheat	Mesosulfuron + iodosulfuron applied annually
ALOMY-004	Waterford	52°22′ N–7°42′ W	2020	NIT	Spring barley	None
			2019	NIT	Spring barley	None
			2018	NIT	Spring barley	None
			2017	Plough	Winter wheat	Mesosulfuron + iodosulfuron
			2016	Plough	Spring oats	None
S-006	Dublin	53°56′ N–6°29′ W	2020	Plough	Winter oats	None
			2019	Plough	Winter wheat	Mesosulfuron + iodosulfuron
			2018	Plough	Winter oilseed rape	Propaquizafop
					Winter wheat	
			2017	Plough	Spring beans	Mesosulfuron + iodosulfuron
			2016	Plough		Propaquizafop

NIT-non-inversion tillage; Mesosulfuron + iodosulfuron from Pacifica Plus ®.

Table 2. Location and field history of the suspected resistant (LOLMU-001 to LOLMU-004) populations of *L. multiflorum* collected from cereal production fields prior to 2020-harvest for resistance testing.

Population	County	Field Position	Year	Establishment System	Crop	LOLMU-Active Herbicides Applied
LOLMU-001	Tipperary	52°35′ N–7°75′ W	2020	NIT	Winter wheat	Pyroxsulam
			2019	NIT	Winter wheat	Mesosulfuron + iodosulfuron
			2018	NIT	Spring oats	None
			2017	NIT	Winter wheat	Mesosulfuron + iodosulfuron
			2016	NIT	Winter wheat	Mesosulfuron + iodosulfuron
LOLMU-002	Cork	52°18′ N–8°84′ W	2020	NIT	Winter wheat	Pyroxsulam
			2019	DD	Spring beans	Cycloxydim
			2018	NIT	Winter barley	Pinoxaden
			2017	NIT	Winter barley	Pinoxaden
			2016	NIT	Winter barley	Pinoxaden
LOLMU-003	Meath	53°48′ N–6°42′ W	2020	Plough	Winter wheat	Mesosulfuron + iodosulfuron
			2019	Plough	Winter wheat	Pyroxsulam
			2018	Plough	Winter wheat	Mesosulfuron + iodosulfuron
			2017	Plough	Winter wheat	Mesosulfuron + iodosulfuron
			2016	Plough	Winter wheat	Pyroxsulam
LOLMU-004	Meath	53°65′ N–6°36′ W	2020	Plough	Winter wheat	Pinoxaden
			2019	Plough	Winter wheat	Pinoxaden
			2018	Plough	Winter oats	None
			2017	Plough	Winter wheat	Pinoxaden
			2016	Plough	Winter wheat	Pinoxaden

NIT-non-inversion tillage; DD-direct drill; Mesosulfuron + iodosulfuron from Pacifica Plus ®; Note: susceptible *L. multiflorum* population SORG-002 was obtained from an organic seed supplier.

Prior to the resistance testing experiments, seed populations of *A. myosuroides* were placed in an incubator at 30 °C for six weeks to break seed dormancy. Subsequently, seeds from *A. myosuroides* and *L. multiflorum* populations were placed in a petri-dish with dense filter paper (VWR ® Grade 413), imbibed overnight in a combination of gibberellic acid (GA_3) and potassium nitrate (KNO_3) solution to assist seed germination [23]. Seeds were then planted in 35.5 × 21.5 × 5 cm plastic trays containing Kettering loam and lime-free grit mix in a 4:1 ratio, with the addition of Osmocote Mini™ (1 kg t^{-1}). Each

population had 35 seeds planted per tray for each replicate. Trays were watered as required throughout the experiment. The plants of *A. myosuroides* and *L. multiflorum* were grown in two separate glasshouse compartments with an 18/12 °C (day/night) temperature regime at a photoperiod of 16 h, supplemented with artificial lighting to maintain a minimum light intensity of 250 µmol quanta m^{-2} s^{-1} at the Teagasc Crops Research Centre, Oak Park, Carlow/Ireland.

2.2. Single-Dose Assays

A range of registered ACCase- and ALS-inhibiting herbicides for *A. myosuroides* and/or *L. multiflorum* control were tested at the recommended application rates (for field application in Ireland) to determine the sensitivity and resistance patterns of the populations of *A. myosuroides* and *L. multiflorum*.

For *A. myosuroides*, two ACCase inhibitors propaquizafop (Falcon ®, Adama) at 100 g active substance (a.s) ha^{-1}; and cycloxydim (Stratos Ultra ®, BASF) at 200 g a.s ha^{-1} and one ALS inhibitor mesosulfuron + iodosulfuron (Pacifica Plus ®, includes amidosulfuron, Bayer CropScience Ltd., Cambridge, UK) at 15 + 5 g a.s ha^{-1} plus 1% *v*/*v* Biopower adjuvant (6.7% *w*/*w* 3, 6-dioxaeicosylsulphate sodium salt (EAC1) and 20.1% *w*/*w* 3, 6-dioxaoctadecylsulphate sodium salt, Bayer), were applied. For *L. multiflorum*, three ACCase inhibitors pinoxaden (Axial Pro ®, includes cloquintocet-mexyl, Syngenta) at 30.3 g a.s ha^{-1} plus 1% *v*/*v* Adigor adjuvant (47% *w*/*w* methylated rapeseed oil, Syngenta); propaquizafop at 150 g a.s ha^{-1} and cycloxydim at 150 g a.s ha^{-1} and two ALS inhibitors mesosulfuron + iodosulfuron at 15 + 5 g a.s ha^{-1} plus 1% *v*/*v* Biopower adjuvant and pyroxsulam (Broadway star ®, includes florasulam, Corteva Agrisciences) at 18.8 g a.s ha^{-1} plus 1% *v*/*v* Kantor adjuvant (EC 790 g L^{-1} alkoxylated triglycerides, Interagro Ltd., Bishop's Stortford, UK) were applied.

Herbicides were applied to the plants at growth stage (GS) 12–13 (BBCH) [24], using a Generation III Research Track Sprayer (DeVries Manufacturing, Hollandale, MN, USA) with a Teejet 8002-EVS flat fan nozzle, at a pressure of 2.5 bar and a water volume equivalent of 200 L ha^{-1}. The experiment was a randomized design with two replicates, a minimum of 27 plants per population for each replicate of each herbicide treatment and was repeated twice. In addition to untreated controls, susceptible populations S-006 for *A. myosuroides* and SORG-002 for *L. multiflorum* were included. At 28 days after spraying, plants were rated as dead (susceptible) or alive (resistant). Above-ground shoots were harvested, dried at 70° C for 72 h and weighed. Plant survival was assessed for each population at each herbicide treatment by expressing the percentage of surviving seedlings. The above ground shoot dry weight for each replicate was expressed as the percentage of the mean dry weight of the untreated control replicates of the corresponding population.

2.3. Dose-Response Assays

Dose-response assays on all populations were carried out to quantify the level of resistance or sensitivity to ACCase- and ALS-inhibiting herbicides. When seedlings reached the 2–3 leaf growth stage, three independent dose-response experiments for *A. myosuroides* and five independent dose-response experiments for *L. multiflorum* were established (Table 3). The selected herbicide dose rates represent 0.25 to 8 times the recommended field rate in Ireland. Each experiment was randomized with three replicates, a minimum of 26 plants per population per replicate for each dose. Above-ground shoot dry weights were harvested and measured 28 days after spraying using methodology above.

Table 3. ACCase and ALS inhibitor dose-response herbicide treatments.

Mode of Action	Chemical Families	Herbicide	Dose (g Active Substance ha^{-1})
			Alopecurus myosuroides
ACCase	FOP	Propaquizafop	25, 50, **100**, 150, 200, 400, 800 and 0
	DIM	Cycloxydim	50, 100, **200**, 300, 400, 800, 1600 and 0
ALS	SU	Mesosulfuron + iodosulfuron	3.8 + 1.3, 7.5 + 2.5, **15 + 5**, 22.5 + 7.5, 30 + 10, 60 + 20, 120 + 40 and 0
			Lolium multiflorum
ACCase	DEN	Pinoxaden	7.6, 15.1, **30.3**, 45.4, 60.5, 121, 242 and 0
	FOP	Propaquizafop	37.5, 75, **150**, 225, 300, 600, 1200 and 0
	DIM	Cycloxydim	37.5, 75, **150**, 225, 300, 600, 1200 and 0
ALS	SU	Mesosulfuron + iodosulfuron	3.8 + 1.3, 7.5 + 2.5, **15 + 5**, 22.5 + 7.5, 30 + 10, 60 + 20, 120 + 40 and 0
	TP	Pyroxsulam	4.7, 9.4, **18.8**, 28.1, 37.5, 75.0, 150.8 and 0

The recommended Ireland field rate for each herbicide is highlighted in bold.

2.4. Analysis of Target-Site Resistance Mutations

Prior to collecting leaf samples to detect TSR mutations within the *ACCase* and *ALS* genes, suspected resistant plants at the 2–3 leaf growth stage (15 plants per population) were sprayed with ALS-inhibiting herbicide mesosulfuron + iodosulfuron (15 + 5 g a.s. ha^{-1}) and additionally, *A. myosuroides* plants were sprayed with ACCase-inhibiting herbicide cycloxydim (200 g a.s. ha^{-1}), all applied at recommended field rates. Eight leaf blades from eight individual plants per resistant population that had survived herbicide treatments, and from untreated control plants in susceptible populations, were harvested 21 days after spraying and kept at −20 °C until used. Genomic DNA was extracted from a total of 80 leaf samples with the MagMAX ™ Plant DNA Kit using the Thermo Scientific ™ Pharma Kingfisher ™ flex 96 Deep-well Head Magnetic Particle Processor. The DNA concentration of each sample was determined using the Quanti-iT ™ PicoGreen ™ dsDNA Assay kit (Invitrogen ™). Then dilutions were made at 40 ng µL^{-1}. A sealed elusion plate, containing frozen DNA, was placed upright in a Styrofoam cooler surrounded by cold ice packs, and sent to IDENTXX, Stuttgart, Germany for TSR genotyping using pyrosequencing. Known homozygous and heterozygous point mutations in the *ACCase* gene at position Ile-1781, Trp-2027, lle-2041, Asp-2078 and Gly-2096, and in the *ALS* gene at position Pro-197 and Trp-574 were analyzed Sequencing and data analysis were performed as described by Keshtkar et al. [25].

2.5. Statistical Analysis

Survival data and shoot dry weight data for single-dose assays of *A. myosuroides* and *L. multiflorum* populations were analyzed separately using ANOVA in R (v.3.6.3), to determine treatment-by-experiment interactions (two replicates per experiment). The interactions for percent survival or percent shoot dry weight was not significant ($p < 0.05$). Therefore, the data was averaged over the four replicates in each of *A. myosuroides* and *L. multiflorum* populations.

Dose-response models were fitted to the shoot dry weight data using the *drc* package [26] in R (v.3.6.3). Lack-of-fit *F*-tests were performed to assess model fit ($p > 0.05$). For *A. myosuroides*, a four-parameter log-logistic model was used to model the propaquizafop and cycloxydim shoot dry weight data, and a four-parameter Weibull-1 model to model the mesosulfuron + iodosulfuron shoot dry weight data. As residuals were normally distributed, data were not transformed. For *L. multiflorum*, a four-parameter log-logistic model with a Box-Cox transformation was used to model the pinoxaden, propaquizafop and cycloxydim shoot dry weight data, and a four-parameter Weibull-1 model with a

Box-Cox transformation to model the mesosulfuron + iodosulfuron and pyroxsulam shoot dry weight data. As virtually complete control of a susceptible population S-006 for *A. myosuroides* or SORG-002 for *L. multiflorum* was achieved at the lowest dose (0.25x field rate) used for most herbicides, a model could not be fitted to these populations. Fitted models estimated the growth rate GR_{50} (i.e., the effective dose required to obtain a growth reduction of 50% relative to untreated plants) and resistance factor (RF) calculated as the ratio between the GR_{50} values of each resistant population by the GR_{50} values of the susceptible control.

3. Results

3.1. Single-Dose Assays

The susceptible *A. myosuroides* population S-006 and the susceptible *L. multiflorum* population SORG-002 were totally controlled by all herbicides tested at recommended field rate (Figures 1 and 2).

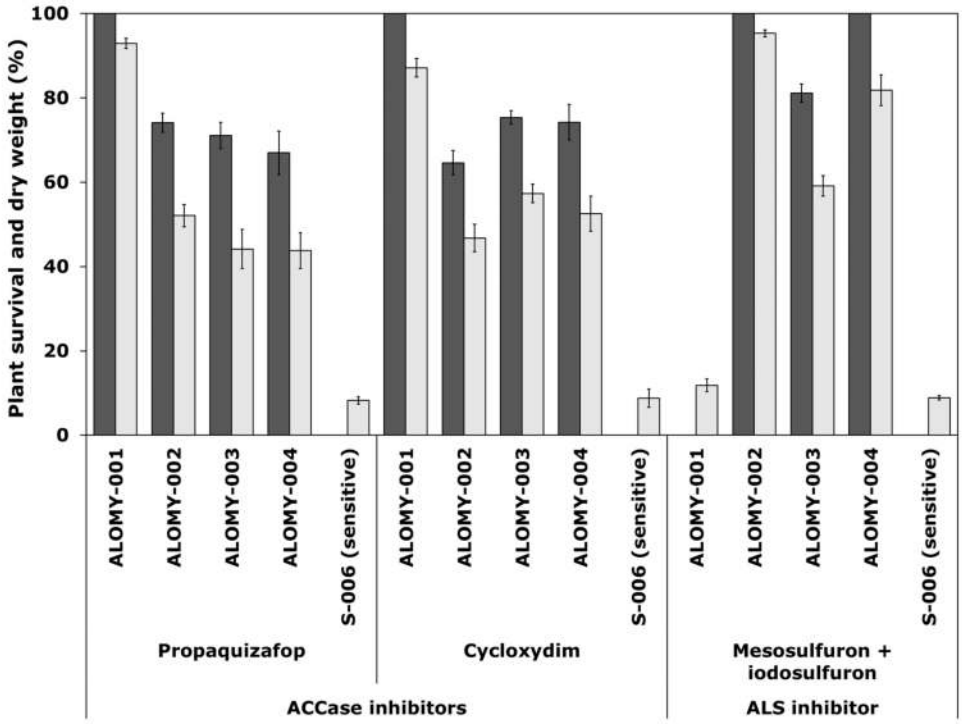


Figure 1. Survival (black) and shoot dry weight (grey), expressed as a percentage of untreated controls of *A. myosuroides* populations. These were treated with two ACCase-inhibiting herbicides propaquizafop (100 g a.s ha^{-1}) and cycloxydim (200 g a.s ha^{-1}) and one ALS-inhibiting herbicide mesosulfuron + iodosulfuron (15 + 5 g a.s ha^{-1}), all applied at recommended Ireland field rate. Error bars are standard error of the mean.

For *A. myosuroides*, resistance was recorded in suspected resistant population ALOMY-001 to both ACCase-inhibiting herbicides propaquizafop (100% survival) and cycloxydim (100% survival), but was totally controlled by the ALS-inhibiting herbicide mesosulfuron + iodosulfuron (Figure 1). Resistance was recorded in the other three suspect populations ALOMY-002, ALOMY-003 and ALOMY-004 to both MOA, with a plant survival rate of 65% to 75% to ACCase inhibitors, respectively, and 81% or 100% to an ALS inhibitor.

For *L. multiflorum*, resistance was recorded in all four suspect populations (LOLMU-001 to LOLMU-004) to both ALS-inhibiting herbicides mesosulfuron + iodosulfuron (100% survival) and pyroxsulam (62–74% survival) (Figure 2). LOLMU-001 and LOLMU-004 were totally controlled by all three ACCase-inhibiting herbicides pinoxaden, propaquizafop and cycloxydim. Whereas, with LOLMU-002 resistance or variation in sensitivity to pinoxaden

and propaquizafop (73% or 43% survival) was recorded, and similarly in LOLMU-003 to pinoxaden (51% survival) and propaquizafop (3% survival only, which is still susceptible), but cycloxydim was found to be effective on both. The results from shoot dry weight data were similar to that of plant survival for the two species (Figures 1 and 2).

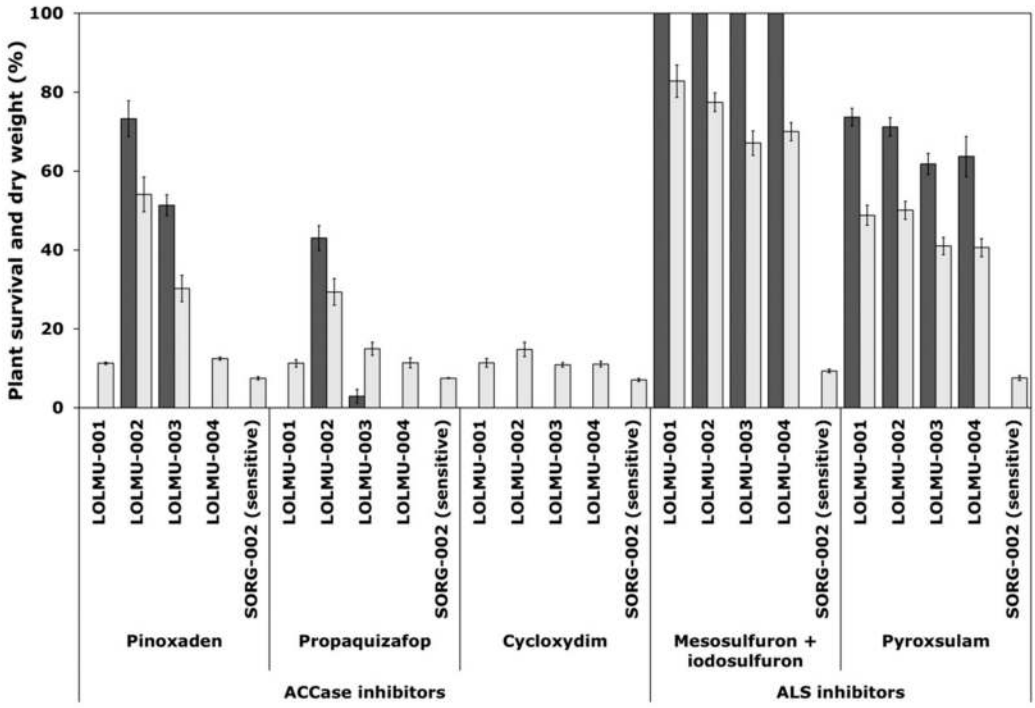


Figure 2. Survival (black) and shoot dry weight (grey), expressed as a percentage of untreated controls of *L. multiflorum* populations. These were treated with three ACCase-inhibiting herbicides pinoxaden (30.3 g a.s ha^{-1}) propaquizafop (150 g a.s ha^{-1}) and cycloxydim (150 g a.s ha^{-1}) and two ALS-inhibiting herbicides mesosulfuron + iodosulfuron (15 + 5 g a.s ha^{-1}) and pyroxsulam (18.8 g a.s ha^{-1}), all applied at recommended Ireland field rate. Error bars are standard error of the mean.

3.2. Dose-Response Assays

Shoot dry weight dose-response models confirmed that there were significant differences in response ($p < 0.01$) amongst the *A. myosuroides* or *L. multiflorum* populations in GR_{50} when treated with ACCase- and ALS-inhibiting herbicides. Model fitting confirmed the extent of resistance, depending on the population and the herbicide, which had been identified in the single-dose assays.

The susceptible populations S-006 for *A. myosuroides*, and SORG-002 for *L. multiflorum* were well controlled, with shoot dry weight reduced by >93% at the recommended field rate of both ACCase and ALS-inhibiting herbicides. In contrast, even though the highest dose rate (i.e., 8x field rate) did not cause complete mortality, shoot dry weight data revealed that the growth of the suspect resistant populations (ALOMY or LOLMU) was clearly affected as the dose rate increased, with more pronounced effects observed from herbicide dose rate equal to or above 1.5× field rate (Figures 3 and 4).

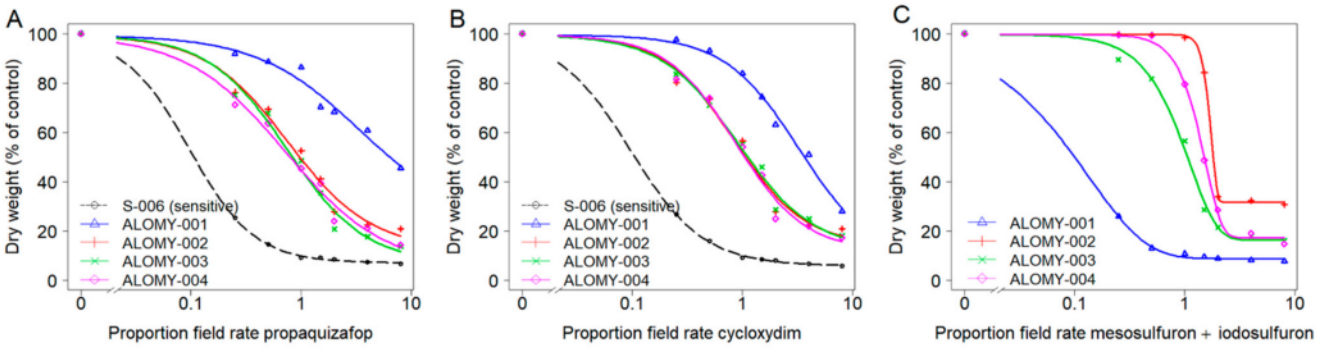


Figure 3. Dose-response analysis of *A. myosuroides* populations for two ACCase-inhibiting herbicides propaquizafop (**A**) and cycloxydim (**B**) and one ALS-inhibiting herbicide mesosulfuron + iodosulfuron (**C**). Note: The control of susceptible population S-006 at the lowest dose was virtually complete, therefore a model (**C**) could not be fitted with S-006.

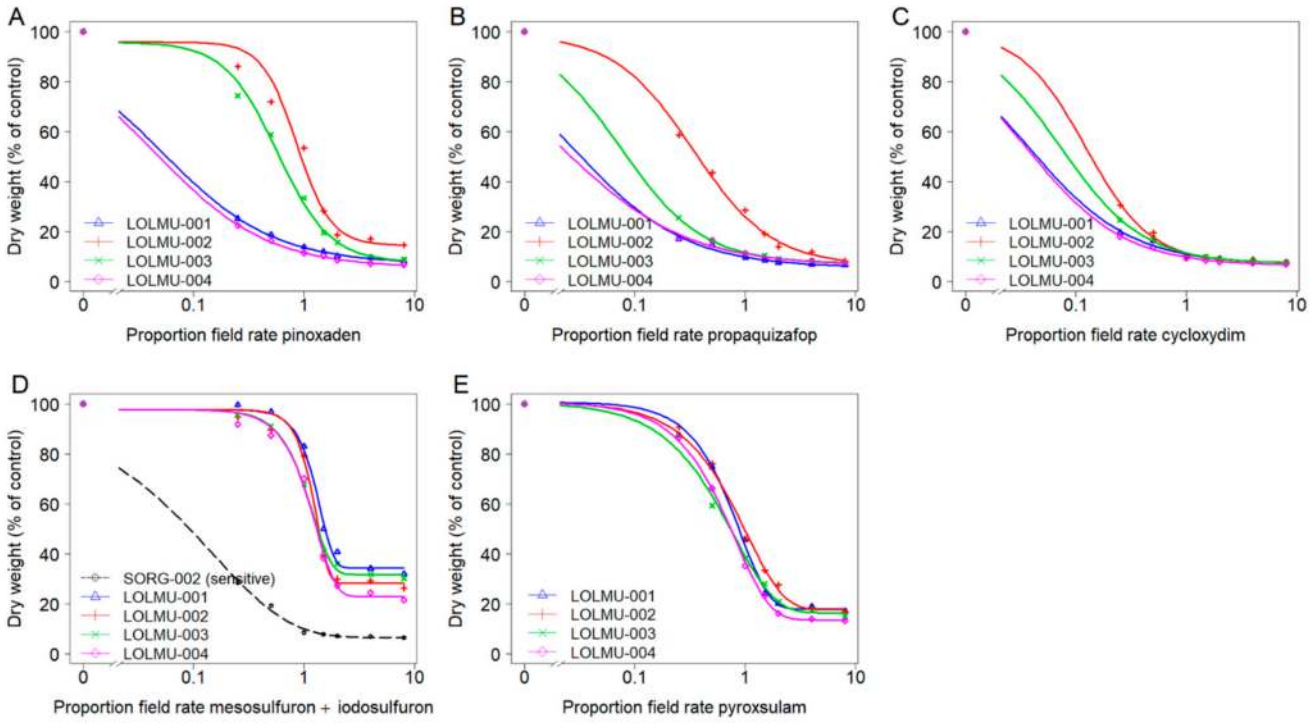


Figure 4. Dose-response analysis of *L. multiflorum* populations for three ACCase-inhibiting herbicides pinoxaden (**A**), propaquizafop (**B**) and cycloxydim (**C**) and two ALS-inhibiting herbicides mesosulfuron + iodosulfuron (**D**) and pyroxsulam (**E**). Note: With the exception of model (**D**), the control of susceptible population SORG-002 at the lowest dose was virtually complete and hence other models could not be fitted with SORG-002.

For *A. myosuroides*, suspect resistant population ALOMY-001 displayed the highest resistance, with the GR_{50} herbicide application rate value estimates being more than 3.2 times the recommended field rate of both ACCase-inhibiting herbicides (Figure 3A,B), and RF was >30 for propaquizafop or cycloxydim (Table 4). In the other three suspect populations ALOMY-002 to ALOMY-004, while their GR_{50} values being slightly below recommended field rate (0.7–0.9), their RF of between 7.5 and 7.9 for propaquizafop, and between 8.7 and 9.3 for cycloxydim, indicated ineffective control with both ACCase-inhibiting herbicides. For the ALS-inhibiting herbicide, GR_{50} values of ALOMY-002 and ALOMY-004 populations were 1.7 or 1.4 times the recommended field rate of mesosulfuron + iodosulfuron, and the GR_{50} values of ALOMY-003 were close to recommended field rate (0.9) (Figure 3C). The RF ranged between 3.6 and 6.6 for mesosulfuron + iodosulfuron (Table 4), indicating

that ALOMY-002, ALOMY-003 and ALOMY-004 were also ALS-resistant. GR_{50} values of ALOMY-001 was similar to S-006, confirming that ALOMY-001 was ALS-sensitive.

Table 4. GR_{50} values (standard errors in parenthesis) from shoot dry weight response to two ACCase inhibitors propaquizafop and cycloxydim and one ALS inhibitor mesosulfuron + iodosulfuron for susceptible (S-006) and resistant (ALOMY-001 to ALOMY-004) populations of *A. myosuroides*. Resistance factor (RF) was calculated as ratio between the GR_{50} of resistant population by the GR_{50} of the susceptible population. ACCase and ALS mutations identified at Ile-1781 or Pro-197 position only. Eight plants were analyzed per population. Number of homozygous and heterozygous *A. myosuroides* plants for specific mutations are reported in the bracket.

	Propaquizafop		**Cycloxydim**		**Mesosulfuron + Iodosulfuron**		**Mutant ACCase**	**Mutant ALS**
Population	**GR_{50} (g a.s ha^{-1})**	**RF**	**GR_{50} (g a.s ha^{-1})**	**RF**	**GR_{50} (g a.s ha^{-1})**	**RF**	**Ile-1781**	**Pro-197**
S-006	9.6 (5.24)	-	19.2 (8.65)	-	<3.8 + <1.3	-	-	-
ALOMY-001	340.6 (297.2)	35.5	653.8 (237.5)	34.1	1.3 (0.6) + 0.4 (0.2)	>0.3	(6/2)	-
ALOMY-002	75.6 (10.56)	7.9	167.1 (17.91)	8.7	25.1 (0.49) + 8.4 (0.16)	>6.6	(5/3)	(1/7)
ALOMY-003	74.2 (8.87)	7.7	178.9 (22.29)	9.3	13.6 (0.52) + 4.5 (0.17)	>3.6	(2/3)	(0/5)
ALOMY-004	71.7 (13.53)	7.5	173.1 (17.8)	9	20.4 (0.49) + 6.8 (0.16)	>5.4	(0/7)	(0/7)

- no known resistance-conferring mutations were found in all tested plants.

For *L. multiflorum*, suspect resistant populations LOLMU-002 and LOLMU-003 were statistically different ($p < 0.05$), compared with the remaining two resistant populations with each of the three ACCase-inhibiting herbicides pinoxaden, propaquizafop and cycloxydim (Figure 4A–C). GR_{50} values of LOLMU-001 and LOLMU-004 were consistent with SORG-002 across ACCase-inhibiting herbicides (Table 5). Whereas, GR_{50} values of LOLMU-002 and LOLMU-003 were slightly below recommended field rate of pinoxaden (0.9 or 0.6), their RF was 3.4 or 2.3, indicating reduced sensitivity. In addition, while GR_{50} values of LOLMU-002 remained well below recommended field rate of propaquizafop (0.3), and RF was also low (1.3), there were some surviving plants, even at the 1.5× field rate, strongly indicating that it is in the process of evolving resistance. Conversely, although LOLMU-003 had a few survivors following propaquizafop application, and both LOLMU-002 and LOLMU-003 had a few survivors to cycloxydim, to dose rates below field rate, they were still well controlled at recommended field rate, with shoot dry weight reduced by >88%. For ALS-inhibiting herbicides, GR_{50} values of LOLMU-001 to LOLMU-004 ranged between 1.0 and 1.3 times the recommended field rate of mesosulfuron + iodosulfuron, whereas\their GR_{50} values were slightly below recommended field rate of pyroxsulam (0.6–0.8) (Figure 4D,E). The RF ranged between 11.9 and 14.6 for mesosulfuron + iodosulfuron, and between 2.3 and 3.1 for pyroxsulam (Table 5) indicating ALS resistance.

Table 5. GR_{50} values (standard errors in parenthesis) from shoot dry weight response to three ACCase inhibitors pinoxaden, propaquizafop and cycloxydim and two ALS inhibitors mesosulfuron + iodosulfuron and pyroxsulam for susceptible (SORG-002) and resistant (LOLMU-001 to LOLMU-004) populations of *L. multiflorum*. Resistance factor (RF) was calculated as ratio between the GR_{50} of resistant population by the GR_{50} of the susceptible population. No known ACCase mutations detected. ALS mutations identified at Pro-197 position only. Eight plants were analyzed per population. Number of homozygous and heterozygous *L. multiflorum* plants for ALS mutation is reported in the bracket.

	Pinoxaden		Propaquizafop		Cycloxydim		Mesosulfuron + Iodosulfuron		Pyroxsulam		Mutant ALS
Population	**GR_{50} (g a.s ha^{-1})**	**RF**	**GR_{50} (g a.s ha^{-1})**	**RF**	**GR_{50} (g a.s ha^{-1})**	**RF**	**GR_{50} (g a.s ha^{-1})**	**RF**	**GR_{50} (g a.s ha^{-1})**	**RF**	**Pro-197**
SORG-002	<7.6	-	<37.5	-	<37.5	-	1.3 (0.24) + 0.4 (0.08)	-	<4.7	-	-
LOLMU-001	1.6 (0.54)	>0.2	4.3 (1.68)	>0.1	5.7 (1.79)	>0.2	19 (0.63) + 6.3 (0.21)	14.6	13.6 (0.65)	>2.9	(6/2)
LOLMU-002	25.9 (1.92)	>3.4	49.7 (4.65)	>1.3	18.3 (1.61)	>0.5	17.8 (0.52) + 5.9 (0.17)	13.7	14.5 (0.74)	>3.1	(3/1)
LOLMU-003	17.3 (1.15)	>2.3	11.1 (2.39)	>0.3	10.9 (1.85)	>0.3	15.5 (0.64) + 5.2 (0.21)	11.9	10.8 (0.6)	>2.3	-
LOLMU-004	1.4 (0.48)	>0.2	3.3 (1.63)	>0.1	5.4 (1.84)	>0.1	16.5 (0.6) + 5.5 (0.2)	12.7	11.8 (0.56)	>2.5	(5/0)

- no known resistance-conferring mutations were found in all tested plants.

3.3. Analysis of Target-Site Resistance Mutations

Pyrosequencing analysis discovered one of the five mutated positions, i.e., Ile-1781 in the *ACCase* gene, in all four suspect resistant (ALOMY-001 to ALOMY-004) populations of *A. myosuroides* (Table 4). In ALOMY-001, six individuals were homozygous for the Ile-1781-Leu substitutions, and two were heterozygous Ile-1781-Ile/Leu. More homozygous mutant plants might have conferred stronger resistance to recommended field rate of propaquizafop and cycloxydim in this population. In ALOMY-002 to ALOMY-004, variability of the ratio between homo/heterozygous, or only heterozygous mutant individuals might have conferred a relatively lower level of resistance to recommended field rate of both ACCase-inhibiting herbicides. Additionally, ALOMY-002 to ALOMY-004 also had the Pro-197 mutation in the *ALS* gene (Table 4). Heterozygous mutant plants (Pro-197-Pro/Thr in ALOMY-002 and ALOMY-004, or combination of Pro-197-Pro/Thr and Pro-197-Pro/Ser in ALOMY-003) were predominant and only one from ALOMY-002 was homozygous (Pro-197-Thr), all of which were sufficient to confer mesosulfuron + iodosulfuron failure at recommended field rate. ALOMY-001 had no mutations at Pro-197 or Trp-574 and herbicide screening also confirmed similar sensitivity as S-006 to mesosulfuron + iodosulfuron.

For *L. multiflorum*, different substitutions for the Pro-197 position in the *ALS* gene were identified in suspect populations LOLMU-001, LOLMU-002 and LOLMU-004 only (Table 5). Predominantly homozygous mutant plants (Pro-197-Gln in LOLMU-001 and LOLMU-002 or Pro-197-Leu in LOLMU-004) conferred resistance to recommended field rate of mesosulfuron + iodosulfuron and pyroxsulam. However, the resistance levels were stronger with mesosulfuron + iodosulfuron, compared with pyroxsulam. Despite no individuals possessing any of the known *ACCase* gene mutations in LOLMU-001 to LOLMU-004, it is interesting that LOLMU-002 was able to survive pinoxaden and propaquizafop, and LOLMU-003 to pinoxaden only, among these populations. In addition, phenotypic resistance to both ALS-inhibiting herbicides was noted in LOLMU-003, but without any known *ALS* gene mutations. These results indicate the likely presence of NTSR mechanisms for ACCase and/or ALS resistance in LOLMU-002 and LOLMU-003. The susceptible *A. myosuroides* (S-006) and *L. multiflorum* (SORG-006) populations had no mutations in any of the known positions in the *ACCase* and *ALS* genes.

4. Discussion

This study is the first to identify and characterize resistance to different ACCase- and ALS-inhibiting herbicides in populations of four *A. myosuroides* (ALOMY-001 to ALOMY-004) and four *L. multiflorum* (LOLMU-001 to LOLMU-004) from Ireland. For *A. myosuroides*, the homo/heterozygous combinations or only heterozygous Ile-1781 mutation was associated with resistance to ACCase-FOP-propaquizafop as well as to ACCase-DIM-cycloxydim in all four ALOMY populations, and the independent Pro-197 mutation (mostly in heterozy-

gous status) was associated with resistance to the ALS-SU-mesosulfuron + iodosulfuron, additionally in ALOMY-002, ALOMY-003, and ALOMY-004. For *L. multiflorum*, all four LOLMU populations were resistant to ALS-SU-mesosulfuron + iodosulfuron as well as to ALS-TP-pyroxsulam, but the Pro-197 mutation (mostly in homozygous status) was only identified in LOLMU-001, LOLMU-002 and LOLMU-004. Despite no known ACCase mutations in these LOLMU populations, LOLMU-002 was able to survive pinoxaden and propaquizafop application and LOLMU-003 was able to survive pinoxaden, but both were cycloxydim-susceptible. These results suggest the presence of NTSR mechanisms for the difference between the genetic and phenotypic data in these two populations. In general, as both homo/heterozygous mutant plants carrying the Ile-1781 or Pro-197 had their growth impacted by higher herbicide dose rate (Figures 3 and 4), and as not all the ACCase/ALS survivors especially in ALOMY-003 and ALOMY-004, or in LOLMU-002 to LOLMU-004 had substitutions (Tables 4 and 5), the resistance recorded was classified as low to moderate in ALOMY and LOLMU.

Diverse mutated positions of ACCase (Ile-1781, Trp-1999, Trp-2027, Ile-2041, Asp-2078, Cys-2088, Gly-2096) and ALS (Pro-197, Asp-376, Trp-574, Ala-122, Ala-205 and Ser-653) conferring TSR have been identified in grass weed species [13,27–29]. Nevertheless, the Ile-1781 and Pro-197 mutations documented in this study are by far the most common mutation in the UK and in Europe to confer distinct resistance spectrum to different ACCase- and ALS-inhibiting herbicides in *A. myosuroides* and/or *L. multiflorum* [6,11,30–33]. Multiple resistance mechanisms via, TSR and/or NTSR to both MOA within a single field population of *A. myosuroides* and *Lolium* spp., and in predominantly self-pollinating *Avena* spp., and *Anisantha and Bromus* spp. are quite frequent worldwide [11,12,17,34–39]. Especially in the UK, NTSR to ACCase-inhibiting herbicides has been consistently more frequent than TSR mechanisms in *L. multiflorum* [11,40]. This may also be the case with LOLMU-002 and LOLMU-003 in this study, as supported by our preliminary results with pendimethalin assays and glutathione transferase phi (F) class 1 (GSTF1) protein, a reliable biomarker of NTSR in *A. myosuroides* (Onkokesung et al. unpublished).

For *A. myosuroides*, with the exception of ALOMY-001 to ALOMY-004, the remaining eight populations (including sensitive S-006) collected from this nationwide survey, were found to be completely sensitive to all three *A. myosuroides*-active herbicides applied at recommended field rate (0% survival and shoot dry weight reduction of >90%). Moreover, our records suggest that most surveyed growers had *A. myosuroides* incidence only in recent years, and strongly believed that the seeds have entered into their farm through different routes of contamination (e.g., via, second generation (C-2) seed imports from the UK, machinery moving from farm to farm, organic manure imports from other farms, or screenings from local mills) (Vijaya Bhaskar et al. unpublished). The source of *A. myosuroides* infestations (either indigenous or imported from the UK) will have implications for the adoption of weed spread prevention strategies. In general, for TSR to be noticed, such resistance types must have already been present at relatively high frequency [31], and growers have recorded weed control difficulties in the field. Herbicide cross-resistance to the same MOA within a single field such as in ALOMY-001, which was ACCase-resistant only, but was well controlled by mesosulfuron + iodosulfuron; or in LOLMU-001 and LOLMU-004, which were ALS-resistant only, but were well controlled by pinoxaden, propaquizafop and cycloxydim, demonstrates that growers can use alternative herbicide MOA as part of a strategy to eliminate or control these resistant populations. The current advice in the UK is to combine pre-emergence herbicides (and, if possible, use non-ACCase and non-ALS herbicides e.g., propyzamide in winter oilseed rape), with cultural and non-chemical IWM such as delayed autumn drilling, spring cropping, higher seed rates, ploughing, diverse rotations and hand rogueing to manage the soil seedbank of populations that were resistant to single site post-emergent herbicides [31].

However, future *A. myosuroides* control is threatened, where herbicide cross-resistance to the same, and different, MOA or multiple resistance mechanisms occur together, such as ALOMY-002 to ALOMY-004 which exhibit TSR to both ACCase- and ALS-inhibiting

herbicides. Similar observations were noted in LOLMU-002 and LOLMU-003, which exhibited combined ALS TSR and ACCase NTSR resistance or ALS/ACCase NTSR resistance alone. It is interesting that ACCase-DIM applied at the recommended field rate was highly effective on both these populations. A similar observation was also reported by Cocker et al. [40], that the occurrence of resistance to FOP herbicides in *L. multiflorum* does not automatically confer cross-resistance to DIM even though both herbicides are from the ACCase group, confirming the complexity in predicting resistance types. Nevertheless, the use of cycloxydim should not be considered as a sustainable solution, taking into account the resistance mechanisms and the high potential of *L. multiflorum* for spreading herbicide-resistance traits [7,9,41]. It is interesting that clethodim (rarely used in Ireland to date [22]), which is also an ACCase-DIM herbicide, may still offer effective control of resistant populations carrying complex multiple resistance mechanisms [42], and may provide an opportunity to growers to use this post-emergent herbicide in sequence with propyzamide. However, Yu et al. [43] showed that, with *Lolium* strains, a number of different ACCase TSR mutations, including Ile-1781, confer clethodim resistance. In practical terms, rotational restriction of winter oilseed rape (recommended one crop of oilseed rape in five years), restricts the effectiveness of these alternative herbicides.

In general, long-term field experiments in the UK have shown that herbicide mixture, rotation or even non-chemical/cultural IWM will have only very little impact on populations with evolved resistance via multiple resistance mechanisms [17,31]. This is because evidence indicates that, at least in the case of TSR, once it is present in a population it does not disappear, even if the selection pressure is removed [44]. Economic models have estimated annual costs of around €464 million for managing herbicide-resistant *A. myosuroides* in the UK [45]. Therefore, it is critical to prevent the development of resistance, both TSR and NTSR, rather than trying to manage resistance once it has appeared in a population [46]. As Ireland is at a different point in resistance evolution to neighboring countries, due to its evolving cropping systems from a mixed farming base, lessons must be learned from other countries' experience. Immediate adoption of a zero-tolerance approach and extreme IWM measures, such as whole cropping (cutting, baling and removing the affected straw), use of grass leys or fallows to prevent a significant soil weed seed bank should be considered for challenging weeds.

5. Conclusions

Using glasshouse experiments and molecular diagnostics to detect TSR mechanism, we confirmed the first cases of cross- and multiple resistance to ACCase- and ALS-inhibiting herbicides in four suspected resistant populations of *A. myosuroides* (ALOMY-001 to ALOMY-004) and *L. multiflorum* (LOLMU-001 to LOLMU-004) from Ireland. In *A. myosuroides*, the homo/heterozygous or only heterozygous Ile-1781 ACCase and Pro-197 ALS TSR mutations was found to be associated with ACCase/ALS resistance. While in *L. multiflorum*, even though mostly homozygous Pro-197 ALS TSR mutation was found to be associated with ALS resistance, there were ACCase/ALS-resistant populations without TSR, suggesting the possibility of NTSR. Mutation rate, level of heterozygosity and herbicide rates were critical for resistance in ALOMY and LOLMU, being classified as low to moderate. Overall, our findings, by definitively determining the resistance status of *A. myosuroides* and *L. multiflorum,* will increase awareness among researchers, agronomists and growers about the need to reduce the reliance on ACCase and ALS herbicides. These results will stress the importance of integrating cultural/non-chemical IWM with farm biosecurity measures, such as machinery hygiene, avoiding straw and manure imports, using C-1 certified seeds, as prevention and control strategies. The current study confirms the need for resistance monitoring in *A. myosuroides* and *L. multiflorum* to ensure that resistance problems do not increase in this region. Knowledge of resistance levels and cross-resistance among the main herbicide types is a valuable information tool to inform intervention strategies to slow or prevent TSR and NTSR resistance evolution in important grass weeds.

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