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Multiple Resistance to Three Modes of Action of Herbicides in a Single Italian Ryegrass (*Lolium multiflorum* L.) Population in China

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Abstract: Italian ryegrass (*Lolium multiflorum* L.), a cross-pollinated grass, is gradually becoming a predominant weed in wheat fields in China and is evolving resistance to many groups of herbicides. The aim of this study is to determine the resistance levels of a single *L. multiflorum* population from a wheat field in Henan Province China, to three modes of action (MoAs) of herbicides and to further characterize the potential resistance mechanisms. This *L. multiflorum* population evolved multiple herbicide resistances to pyroxsulam [acetolactate synthase (ALS)], pinoxaden [acetyl-CoA carboxylase (ACCase)] and isoproturon [photosystem II (PSII)]. Target-site resistance (TSR) mutations (Pro-197-Gln, Pro-197-Thr, and Trp-574-Leu) and non-target-site resistance (NTSR) mediated by cytochrome P450 monooxygenase (CYP450) genes were associated with pyroxsulam resistance. Pinoxaden resistance was conferred by two TSR mutations, which referred to a rare Ile-2041-Val mutation and a common Ile-1781-Leu mutation but with two different nucleotide substitutions (CTA/TTA). CYP450- and glutathione-S-transferase (GST)-mediated resistances were the main resistance mechanisms for this multiple herbicide-resistant (MHR) population to the PSII inhibitor isoproturon. This is the first case of a single *L. multiflorum* population evolving multiple resistance to three herbicide MoAs (ALS, ACCase and PSII) in China. Diverse resistance mechanisms including TSR and NTSR mean *L. multiflorum* exhibits a high degree of resistance plasticity.

Keywords: *Lolium multiflorum* L.; pyroxsulam; pinoxaden; isoproturon; multiple herbicide resistance; TSR; CYP450; GST

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1. Introduction

Herbicide application provides production security for agriculture; meanwhile, the development of herbicide-resistant (HR) weeds poses a considerable challenge as herbicide selection pressure accumulates. Currently, a total of 515 unique cases of HR weeds (species combined with modes of action [MoAs]) to 21 of 31 known herbicide MoAs and to 165 different herbicides have been documented in 267 species globally [1]. Of these, resistance to herbicides with multiple MoAs has also been reported and raised substantial concerns. Resistances to multiple herbicide MoAs are documented in some malignant weeds such as rigid ryegrass (*Lolium rigidum* Gaudin) [2], black grass (*Alopecurus myosuroides*) [3], *Echinochloa* species [4,5], *Amaranthus* species [6,7], and Italian ryegrass (*Lolium multiflorum* L.) [8].

L. multiflorum is a cross-pollinated plant that was originally grown as a forage grass [9]. It was introduced to China in the 1930s for lawn and pasture cultivation [10]. However, it has gradually expanded to wheat fields in China and impacted wheat production [11–13]. At present, *L. multiflorum* control relies on chemical herbicides, including acetolactate synthase (ALS)-, acetyl coenzyme A carboxylase (ACCase)- and photosystem II (PSII)-inhibiting herbicides, represented in this study by pyroxsulam, pinoxaden and isoproturon, respectively.

ALS inhibitors inhibit the de novo synthesis of branched-chain amino acids [14–16]. Up to now, five major chemical families of ALS inhibitors have been developed: sulfonylureas (SUs), imidazolinones (IMIs), pyrimidinothiobenzoates (PTBs), triazolopyrimidines (TPs) and sulfonylcarbonyltriazolinones (SCTs) [1]. The ACCase inhibitors disrupt the biosynthesis of fatty acid and are divided into three herbicide chemical families: aryloxyphenoxypropionates (APPs), cyclohexanediones (CHDs), and phenylpyraxoline (PPZ) [17]. Although isoproturon was banned in some countries due to its harmful effects on the environment [18], as another herbicide MoA, isoproturon has proven to be an effective alternative for managing a variety of troublesome weeds resistant to ALS and/or ACCase inhibitors [19–24], and is one of the important options for weed control in wheat fields in China. Isoproturon belongs to the PSII inhibitors, which interfere with electron transfer in photosynthesis by binding with D1 protein, thus blocking the carbon cycle and ultimately leading to weed death.

There are two primary mechanisms by which weeds develop herbicide resistance: target-site-resistance (TSR) and non-target-site resistance (NTSR) [14,25,26]. The TSR mutations in target genes that confer weeds' resistance against ALS-, ACCase- and PSII-inhibiting herbicides have been well characterized. So far, more than 30 amino acid (AA) substitutions at nine positions in *ALS* (Ala-122, Pro-197, Ala-205, Phe-206, Asp-376, Arg-377, Trp-574, Ser-653 and Gly-654) have been identified as capable of endowing resistance to ALS inhibitors [27,28]. Likewise, at seven positions (Ile-1781, Trp-1999, Trp-2027, Ile-2041, Asp-2078, Cys-2088, Gly-2096) in the conserved region of *ACCase*, at least 13 different AA substitutions have been confirmed to result in resistance to ACCase inhibitors [29]. In *psbA*, nine AA substitutions known to confer resistance to PSII inhibitors at seven positions (Leu-218, Val-219, Ala-251, Phe-255, Ser-264, Asn-266 and Phe-274) have been reported [26,30].

Apart from many identified mutations associated with TSR to the three herbicide MoAs, NTSR has been widely investigated [26]. An NTSR mechanism confers weed resistance by reducing the herbicide reaching the target site, including reduction of herbicide absorption and transportation, enhanced herbicide metabolism (EHM), and herbicide isolation [31]. Among these, EHM has been extensively explored. A series of metabolic enzymes are involved in EHM, including cytochrome P450 monooxygenase (CYP450), glutathione-S-transferase (GST), glycosyltransferase (GT), ATP-binding cassette (ABC) transporter, and other enzyme systems capable of metabolizing herbicides, such as oxidases and hydrolases [32]. EHM potentially leads to more dire consequences for weed control than TSR, as HR weeds with metabolic resistance tend to develop resistance to herbicides with multiple MoAs, and may even survive new herbicide MOAs [31,33].

Farmers prefer to increase herbicide dosage or choose single herbicides to eliminate weeds, but this management practice creates a high level of selection pressure which can lead to weeds evolving high level of herbicide resistance. Resistance to the SU herbicide mesosulfuron-methyl from ALS inhibitors and the APP herbicide clodinafop-propargyl as well as fenoxaprop-p-ethyl from ACCase inhibitors in China has been previously confirmed [13,34–36]. However, the resistance risk and potential resistance mechanisms to the TP herbicide pyroxsulam, PPZ herbicide pinoxaden and PSII inhibitor isoproturon remain unknown. Therefore, an *L. multiflorum* population from Henan Province, China, with suspected multiple herbicide resistance to all three herbicides, was collected to determine the level of resistance. Our current studies on this population are expected to clarify the following issues: i. determining the status of resistance to pyroxsulam, pinoxaden and isoproturon; ii. exploring the resistance mechanisms to three herbicide MoAs; iii. investigating the cross- and multiple-resistance profiles.

2. Materials and Methods

2.1. Plant Materials

In 2020, *L. multiflorum* seeds were collected at maturity which were suspected to be multiple-herbicide resistant from a wheat field in Henan Province (DH-HZYC-2020-1, here-

after referred to as MHR), China, where ALS, ACCase and PSII inhibitors had been applied for more than 5, 10 and 5 years, respectively. Furthermore, seeds of a sensitive population (DH-JNXW-2020-2, hereafter referred to as HS) were collected from a recreational site with no record of herbicide application in Jiangsu Province (Table 1). To maintain their biological activity, seeds were collected manually from 50 mature plants per population, dried and subsequently stored in paper bags under suitable conditions (4 °C, ventilation) for later use.

Table 1. Geographical information for the two *L. multiflorum* populations used in this study.

Population	Suspected Biotype [a]	Geographical Location		
		Longitude	Latitude	Province
DH-JNXW-2020-2	HS	118.87° E	32.03° N	Jiangsu Academy of Agricultural Sciences, Xuanwu District, Nanjing, Jiangsu Province
DH-HZYC-2020-1	MHR	113.95° E	32.95° N	Dashu Au, Yicheng District, Zhumadian, Henan Province

[a] Abbreviations: HS, herbicide-sensitive; MHR, multiple herbicide-resistant.

2.2. Herbicides and Chemicals

Pyroxsulam, pinoxaden, isoproturon and other 10 herbicides of ALS or ACCase inhibitors used in whole-plant dose–response assays are shown in Table 2. Two CYP450 inhibitors, piperonyl butoxide (PBO, 95%) and malathion (95%), and a GST inhibitor, 4-chloro-7-nitrobenzoxadiazole (NBD-Cl, 97%), were purchased from Aladdin (Shanghai, China, CAS#51-03-6), Jiangsu Fengshan Group Co. Ltd. (Yancheng, Jiangsu, China) and Sigma-Aldrich (Beijing, China, CAS#10199-89-0), respectively.

Table 2. Herbicides and their doses used in whole-plant dose–response assays.

Class [a]	Group [a]	Herbicide	Formulation	Manufacturer	Doses Applied g a.i. ha^{-1} [b]	
					HS	MHR
ALS	TP	Pyroxsulam	7.5% WG	Dow AgroSciences, Beijing, China	0,0.11,0.22,0.44,0.88,1.75,3.5	0,3.5,7,**14**,28,56,112
		Penoxsulam	25g L^{-1} OD	Dow AgroSciences, Beijing, China	0,0.47,0.94,1.88,3.75,7.5,**15**	0,**15**,30,60,120,240
	SU	Mesosulfuron-methyl	30 g L^{-1} OF	Bayer, Hangzhou, China	0,0.42,0.84,1.69,3.38,6.75,**13.5**	0,**13.5**,27,54,108,216
	PTB	Bispyribac-sodium	20% WP	Ruibang Agrochemical, Jiangsu, China	0,0.94,1.88,3.75,7.5,15,**30**	0,**30**,60,120,240,480
	IMI	Imazethapyr	5% AS	Changqing Agrochemical, Jiangsu, China	0,2.81,5.63,11.25,22.5,45,**90**	0,22.5,45,**90**,180,360
		Imazamox	4% AS	Flag Chemical, Jiangsu, China	0,1.41,2.81,5.63,11.25,22.5,**45**	0,11.25,**45**,90,180
	SCT	Flucarbazone-sodium	70% WG	Arysta Life Science, Shanghai, China	0,1,2,4,8,16,**32**	0,**32**,64,128,216,512
ACCase	APP	Fenoxaprop-p-ethyl	69 g L^{-1} EW	Bayer, Hangzhou, China	0,60,120,240,480,960	0,240,480,960,1920, 3840
		Clodinafop-propargyl	15% WP	Syngenta, Shanghai, China	0,2.25,4.5,9,18,**36**	0,18,**36**,72,144,288
	CHD	Sethoxydim	12.5% EC	Changqing Agrochemical, Jiangsu, China	0,3.13,6.25,12.5,25,50,100	0,**200**,400,800,1600, 3200
		Tralkoxydim	40% WG	Jiangsu Agrochem Laboratory Co., Ltd., China	0,0.39,1.56,6.25,25,100,**400**	0,50,100,200,**400**, 800,1600
	PPZ	Pinodexn	5% EC	Syngenta, Shanghai, China	0,1.41,2.81,5.63,11.25,22.5,**45**	0,11.25,22.5,**45**,90,180
PSII	Urea	Isoproturon	50% WP	Jiangsu Futian Agrochemical Co., Ltd., China	0,32.81,65.63,131.25,262.5,525,**1050**	0,65.63,131.25,262.5, 525,**1050**,2100

[a] Abbreviations: ACCase, acetyl CoA carboxylase; ALS, acetolactate synthase; PSII, Photosystem II; TP, triazolopyrimidine; SU, sulfonylurea; PTB, pyrimidinylthiobenzoate; IMI, imidazolinone; SCT, sulfonylaminocarbonyltriazolinoe; APP, aryloxyphenoxypropionate; CHD, cyclohexanedione; PPZ, phenylpyrazoline; AS, aqueous solution; EC, emulsifiable concentrate; EW, emulsion in water; OD, oil dispersion; OF, oil flowable concentrate; OP, organophosphates; WG, water-dispersible granule; WP, wettable powder; SC, suspension concentrate; [b] The recommended field doses of different herbicides are shown in bold.

2.3. Whole-Plant Dose Response

Twenty-five seeds of *L. multiflorum* for each population were evenly sown in plastic pots (7 × 7 × 8 cm) containing substrate (pH = 7.0, 1% organic matter, Jiangsu Xingnong Technology Co., Ltd.) and soil at a ratio of 1:2, with the top layer covered to 0.5 cm height with the same mixture. Subsequently, the pots were transferred to a greenhouse (200 µmol m^{-2} s^{-1} photosynthetic photonflux density, 20 °C/15 °C, 12/12 h day/night, ~75% relative humidity), with water and nutrients maintained. The plants were thinned to 15 plants with consistent growth per pot at the 2-leaf stage and treated with herbicides at the 3-leaf stage. An experimental sprayer (Model: 3WP-2000, Nanjing Research Institute for Agricultural Mechanization, Nanjing, National Ministry of Agriculture of China), equipped with a flat fan nozzle delivering 280 L ha^{-1} water at 230 KPa, was used for spraying herbicides. Herbicide dose gradients were established based on the results of pre-experiments with the recommend doses for HS and MHR populations (data not shown) and are listed in Table 2. After the leaves absorbed the herbicide, the plants were returned back to the greenhouse and rehydrated every two days. The fresh weight of plants on the ground was recorded 21 days after herbicide treatment. For each herbicide dose, four replicates were set. The whole experiment was repeated twice.

The resistance patterns of the MHR population to other herbicides were also determined. Whole-plant dose–response assays were performed as described above, and herbicide application doses are listed in Table 2.

2.4. DNA Sequencing of Target Genes from Three Herbicide MoAs

To ensure the accuracy of sequencing, surviving MHR individuals (20 for pyroxsulam, 10 for pinoxaden and 10 for isoproturon) in the whole-plant dose–response experiment and 10 HS individuals were subjected to a Plant Genomic DNA kit (Tiangen Biotech Co. Ltd., Beijing, China) for DNA extraction. A total of 50 µL of the total polymerase chain reaction (PCR) system included the following components: 25 µL of 2 × PCR Taq Mix, 2 µL of 10 µM forward and reverse primers (Table 3), 2.5 ng of genome DNA, and the remaining volume of ddH_2O. The following steps were taken to set up the PCR reaction program: 95 °C for 3 min, 35 cycles of 95 °C for 15 s, X °C (the annealing temperature of primers) for 15 s and 72 °C for 1 min, and, subsequently, 5 min at 72 °C for a further extension to inactivate DNA polymerase.

Following visualization of the PCR products on 1% agarose gels, a commercial kit (TaKaRa Biotechnology, Dalian, China) was used for purification. The purified fragments were ligated into pMD19-T vectors (TaKaRa Biotechnology, Dalian, China) for cloning. Positive clones were sent for DNA sequencing [37]. To obtain the sequence of target genes, a minimum of eight positive clones from each plant were selected to sequence. The sequencing results for both populations were subjected to BioEdit Sequence Alignment Editor Software (Ibis Bioscience, Carlsbad, CA, USA) for sequence alignment.

Table 3. Primers used in this study.

Primers	Sequence (5′-3′)	Product Size (bp)	Tm (°C)	Usage	Sequence Source
ALS-F	CCGCAAGGGCGCCGACATCCTCGT	1719	62	Sequencing	AF310684.2
ALS-R	CGAAATCCTGCCATCACCTTCCAT				
ACCase-F	AATGGGTCGTGGGGCACTCCTATAATTCC	1600	61	Sequencing	Reference [13]
ACCase-R	CTCCCTGGAGTTGTGCTTTC				
psbA-F	ATGACTGCAATTTTAGAGAGACGC	1023	60	Sequencing	EU360732.1
psbA-R	TAGAGGGAAGTTGTGAGCAT				
RGTP [a]-F	GATGTGACTGACCAAGAGAGCTTCA	117	60	qRT-PCR	Reference [38]
RGTP-R	CTCAGCTAAGTCGCATTTGTTCCCC				
psbA-F	ATTCCAGGCAGAGCACAACAT	156	60	qRT-PCR	EU360732.1
psbA-R	GTAACCCTCATTAGCAGATTCATTT				

[a] Ras family GTPase.

2.5. psbA Gene Expression Analysis

Both *L. multiflorum* populations, HS and MHR, were cultured as previously described in Section 2.3 and treated with 1050 g a.i. ha^{-1} of isoproturon at the 3-leaf stage. Six time points (0 h, 6 h, 12 h, 1 d, 3 d, 5 d) were set to analyze the difference in *psbA* gene expression between MHR and HS. For each time point, 100 mg fresh leaf tissue was cut and subjected to the RNA Simple Total RNA Kit (Tiangen Biotech Co. Ltd., Beijing, China) for RNA extraction. Subsequently, according to the manufacturer's instructions, the cDNAs were synthesized with HiScript II Q RT SuperMix for qPCR (+gDNA wiper; Vazyme Biotech Co. Ltd., Nanjing, China). Primers (Table 3) were designed in the conserved regions of *psbA*, and the expression level of *psbA* was evaluated using RPTG as a housekeeping gene [38]. The RT-qPCR was carried out according to the procedure of Fang et al. [27]. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative changes in gene expression in MHR versus HS [39], and the initial expression of each population (untreated control) was used to eliminate the intrinsic differences between populations. The experiment was performed twice with three biological replicates.

2.6. Effects of CYP450 and/or GST Inhibitors on Isoproturon and Pyroxsulam Resistance

Seeds from both *L. multiflorum* populations, HS and MHR, were cultured with the methods described in Section 2.3 to the 3-leaf stage and treated with isoproturon or pyroxsulam in the absence and presence of CYP450 inhibitors (PBO and malathion) and one GST inhibitor (NBD-Cl). PBO and malathion were used to assay pyroxsulam, and PBO and NBD-Cl were used to assay isoproturon. PBO (4200 g a.i. ha^{-1}), malathion (1000 g a.i. ha^{-1}) and NBD-Cl (270 g a.i. ha^{-1}) were applied 1 h, 1h and 2 d prior to herbicide treatment, respectively. Isoproturon was used at 0, 16.41, 32.81, 65.63, 131.25, 262.5, and 525 g a.i. ha^{-1} to assay HS, and at 0, 65.63, 131.25, 262.5, 525, 1050, and 2100 g a.i. ha^{-1} to assay MHR. Pyroxsulam was used at 0, 0.11, 0.22, 0.44, 0.88, 1.75, and 3.5 g a.i. ha^{-1} to assay HS, and at 0, 3.5, 7, 14, 28, 56, and 112 g a.i. ha^{-1} to assay MHR.

The fresh weight of above-ground plants was recorded 21 days after herbicide treatment. Three replicates were set for each herbicide dose, and the whole experiment was repeated twice.

2.7. Data Analysis

The biomass of above-ground plants from two experiments is presented as the percentage of untreated plants. The data sets were subjected to analysis of variance (ANOVA) in SPSS v. 21.0 (IBM, Armonk, NY, USA). The results of ANOVA showed that no significant difference ($p > 0.05$) was found in plant response between the two replicated trials; therefore, the data were pooled, while herbicide dose had a significant effect ($p < 0.05$) on weed growth. The dose response was nonlinear; thus, the data were fitted and analyzed using the log-logistic model [40]. The herbicide concentration that resulted in a 50% reduction in growth (GR_{50}) was calculated in SigmaPlot v. 10.0 (SigmaPlot Software, Chicago, IL, USA) using a four-parameter nonlinear regression equation, which is shown below.

$$y = c + (d - c)/[1 + (x/g)]^b$$

where y denotes the biomass of above-ground plants at the herbicide dose x, d is the upper limit, c is the lower limit, g is the herbicide rate at the point of inflection halfway between d and c, and b is the slope of the curve. The resistance index (RI) was calculated using the GR_{50} values of the MHR and HS populations.

3. Results

3.1. Resistance to ACCase-Inhibiting Pinoxaden

At the field dose of pinoxaden, high levels of fresh weight reduction (almost 100%) were observed in the HS population, but increased herbicide tolerance was observed in MHR, with a fresh weight of 44% (Figure 1A). The GR_{50} value for MHR was

33.28 g a.i. ha^{-1}, with 3.55 g a.i. ha^{-1} for HS. Compared with HS, the MHR population had a 9.39-fold resistance to pinoxaden based on the GR_{50} ratio of MHR and HS (Table 4).

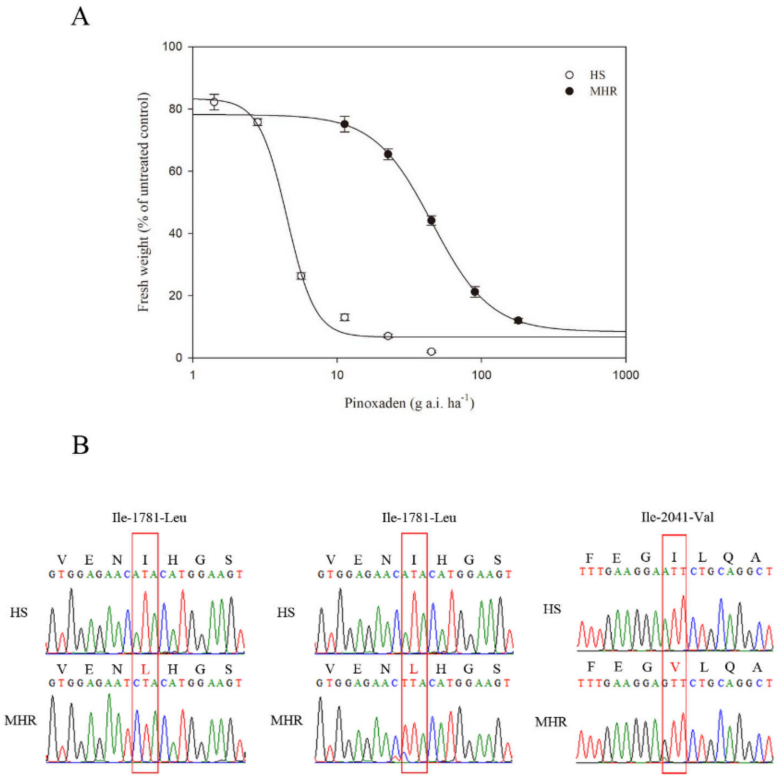

Figure 1. The MHR population is resistant to ACCase-inhibiting pinoxaden. (**A**) Percentage fresh weight with pinoxaden treatment in whole-plant dose–response assay. (**B**) Mutations in *ACCase* gene. Vertical bars represent the mean ± SE. Amino acid sequence positions refer to the sequence of *ACCase* from *Alopecurus myosuroides* Huds. (GenBank accession number AJ310767.1). Non-synonymous mutations are marked with red boxes.

Table 4. Whole-plant dose–response assays.

Herbicides	Populations	Regression Parameters [a]				GR_{50}(SE) [b]	RI [c]
		c	d	b	R^2		
Pyroxsulam	HS	0.26(2.03)	98.47(9.24)	−1.18(0.15)	0.9995	0.24(0.06)	1.00
	MHR	16.26(16.05)	91.06(7.89)	−1.76(0.95)	0.9896	29.22(1.51)	121.75
Pyroxsulam+ PBO	HS	0.88(2.58)	98.15(11.72)	−1.38(0.24)	0.9987	0.20(0.04) [d]	0.83
	MHR	14.33(7.75)	88.86(19.76)	−1.57(0.87)	0.9817	11.65(1.30) *	58.25
Pyroxsulam+ Malathion	HS	−0.73(3.65)	99.81(11.69)	−1.04(0.18)	0.9992	0.21(0.03) [d]	0.88
	MHR	14.33(5.37)	90.62(5.73)	−1.69(0.43)	0.9953	20.48(0.92) *	97.52
Penoxsulam	HS	15.79(12.13)	90.25(1.14)	−1.33(0.41)	0.9957	4.66(1.14)	1.00
	MHR	−8.97(4.43)	94.34(6.60)	−1.12(0.53)	0.9983	161.29(27.73)	34.61
Mesosulfuron-methyl	HS	6.53(4.67)	94.44(11.84)	−1.70(0.50)	0.9941	1.34(0.26)	1.00
	MHR	10.11(18.90)	91.59(17.26)	−1.38(0.87)	0.9938	56.57(16.41)	42.22
Bispyribac-sodium	HS	−13.89(4.92)	97.54(3.31)	−0.99(0.29)	0.9985	29.33(10.42)	1.00
	MHR	−1.83(7.64)	94.02(3.88)	−1.10(0.30)	0.9995	351.96(56.95)	12.00
Imazethapyr	HS	12.02(1.90)	87.32(3.75)	−3.45(0.60)	0.9969	8.66(0.57)	1.00
	MHR	18.66(4.97)	81.49(3.70)	−2.38(0.63)	0.9971	105.34(11.12)	12.16
Imazamox	HS	8.85(1.01)	88.61(3.51)	−2.22(0.22)	0.9994	3.86(0.24)	1.00
	MHR	6.66(6.70)	95.56(5.39)	−2.04(0.49)	0.9980	50.24(5.10)	13.02
Flucarbazone-sodium	HS	2.83(5.74)	97.79(39.16)	−1.15(0.51)	0.9946	1.77(0.25)	1.00
	MHR	4.27(29.31)	82.74(3.75)	−1.37(0.62)	0.9980	407.46(31.24)	230.20

Table 4. *Cont.*

Herbicides	Populations	Regression Parameters [a]				GR_{50}(SE) [b]	RI [c]
		c	d	b	R^2		
Fenoxaprop-P-ethyl	HS	3.42(2.90)	87.84(12.74)	−1.68(0.42)	0.9964	66.01(14.71)	1.00
	MHR	−26.44(5.27)	102.50(1.17)	−1.14(0.06)	0.9996	2030.86 (139.61)	30.77
Clodinafop-propargyl	HS	5.12(2.29)	83.62(9.71)	−1.96(0.44)	0.9965	5.27(0.83)	1.00
	MHR	−16.23(5.52)	93.61(7.83)	−0.95(0.43)	0.9991	232.49(21.57)	44.12
Sethoxydim	HS	4.04(8.68)	83.95(20.41)	−1.72(1.01)	0.9867	8.00(4.81)	1.00
	MHR	6.04(7.00)	88.87(4.07)	−3.06(0.81)	0.9960	1220.73 (122.43)	152.67
Tralkoxydim	HS	1.07(6.02)	100.48(10.87)	−0.91(0.27)	0.9932	5.71(1.89)	1.00
	MHR	8.72(1.71)	90.07(0.83)	−1.46(0.07)	0.9999	578.35(13.54)	101.34
Pinoxaden	HS	6.70(3.04)	83.29(5.53)	−4.47(1.42)	0.9921	3.55(0.44)	1.00
	MHR	8.41(1.16)	78.21(1.03)	−2.16(0.12)	0.9999	33.28(1.01)	9.39
Isoproturon	HS	0.04(1.13)	111.32(14.28)	−1.16(0.12)	0.9997	42.15(9.05)	1.00
	MHR	7.17(1.13)	97.38(3.19)	−2.21(0.17)	0.9998	307.21(12.31)	7.29
Isoproturon+ PBO	HS	−1.04(11.61)	104.53(25.73)	−1.48(0.79)	0.9845	38.32(7.48) [d]	0.91
Isoproturon+ NBD-Cl	HS	−4.53(2.34)	99.68(8.78)	−1.12(0.15)	0.9995	41.09(4.26) [d]	0.97

[a] Parameter values of the four-parameter log-logistic equation $y = c + (d - c)/[1 + (x/g)^b]$. d is the upper limit, c is the lower limit, g is the herbicide rate at the point of inflection halfway between d and c, and b is the slope of the curve. Data were presented as means (SE). [b] GR_{50} means the effective dose of herbicide causing 50% inhibition of fresh weight and is indicated as grams of active ingredient per hectare (g a.i. ha^{-1}). Data were presented as means (SE). [c] RI is the resistance index and is calculated as the ratio between the GR_{50} of the resistant population and the GR_{50} of the susceptible population. Herbicide resistance was classified into five groups: S, not resistant (RI < 2); L, low resistant (RI = 2–5); M, moderate resistant (RI = 6–10); H, high resistant (RI = 11–100); and very high resistance (RI > 100) [41]. [d] No significant differences detected (*t*-test, $p > 0.05$). * Significant differences detected (*t*-test, $p < 0.05$).

Two mutations at seven characterized TSR positions were found in the *ACCase* of MHR population, a common Ile-1781-Leu mutation and a less frequent Ile-2041Val mutation, respectively (Figure 1B). In Ile-1781 allele, two different base substitutions (ATA-CTA/TTA) resulted in the replacement of amino acid Ile by Leu. All 10 survivors showed TSR mutation, 9 individuals were the common Ile-1781-Leu mutation (8 for ATA-TTA substitution and 1 for ATA-CTA substitution), and only one individual carried the less frequent Ile-2041Val mutation.

3.2. Resistance to ALS-Inhibiting Pyroxsulam

Slightly poor biomass reduction (68%) was recorded for MHR population when treated with the ALS-inhibiting herbicide pyroxsulam at the tested highest dose (112 g a.i. ha^{-1}: 8× rate of the field dose), but high biomass inhibition (90%) to HS was observed at 1/8× rate of the field dose of pyroxsulam (Figure 2A). The GR_{50} values for MHR and HS populations were 29.22 g a.i. ha^{-1} and 0.24 g a.i. ha^{-1}, respectively (Table 4). MHR presented 121.75-fold resistance to pyroxsulam based on the RI values.

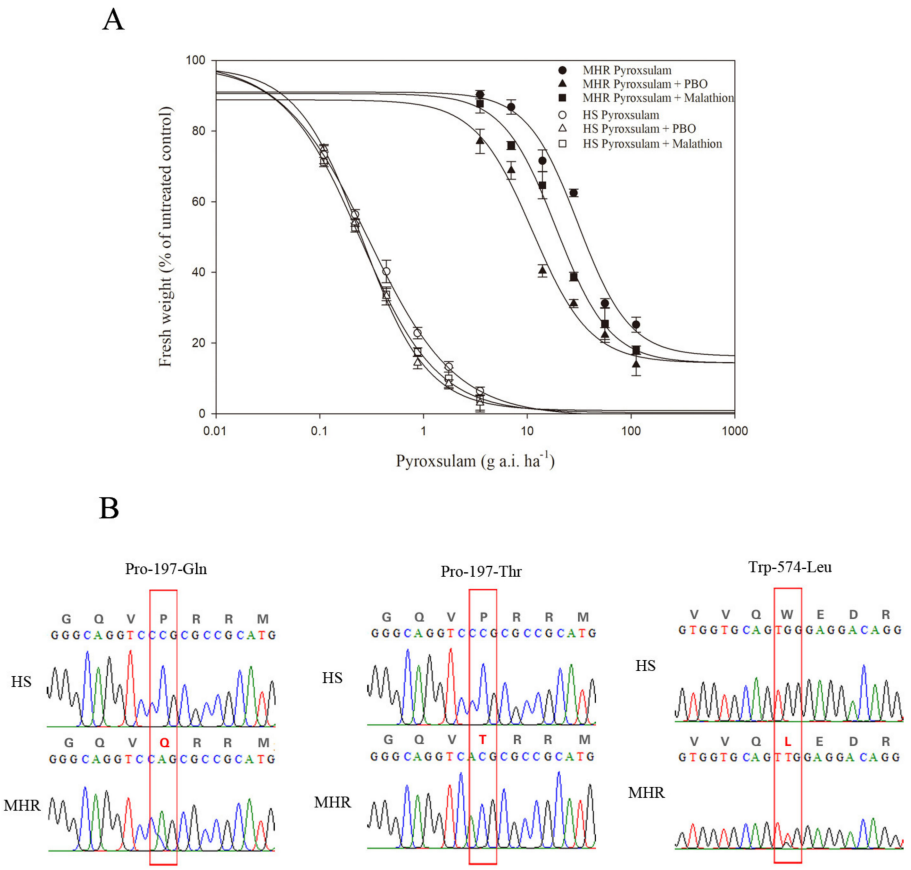

Figure 2. The MHR population has resistance to ALS-inhibiting pyroxsulam. (**A**) Percentage fresh weight with application of pyroxsulam and P450 inhibitors in the whole-plant dose–response assay. (**B**) Mutations in the *ALS* gene. Vertical bars represent the mean ± SE. Amino acid sequence positions refer to the sequence of *ALS* from *Arabidopsis thaliana* (GenBank accession number NM_114714). Non-synonymous mutations are marked with red boxes.

As expected, no known mutations associated with TSR were detected in the plants from HS population. However, sequence analysis on a set of pyroxsulam-survivors revealed that MHR population had two resistant 197 alleles (Thr-197 and Gln-197) and one resistant Trp-574-Leu allele, indicating TSR resistance to pyroxsulam (Figure 2B). Among 20 survivors, 5 individuals were Thr-197 allele (2 homozygous/3 heterozygous), 3 individuals were heterozygous Pro-197-Gln mutation, 3 individuals were heterozygous Trp-574-Leu mutation, and 9 individuals were wild type.

In addition to TSR resistance, NTSR mediated by CYP450 genes played a role in pyroxsulam resistance in the MHR population. Table 4 summarizes the changes in GR_{50} values in the resistant population MHR and the sensitive population HS in the presence or absence of CYP450 inhibitors in detail. As expected, no significant changes were found in the GR_{50} value of the sensitive population (*t*-test, $P > 0.05$), indicating that CYP450-mediated metabolism did not exist in this sensitive population. However, co-application of pyroxsulam with the CYP450 inhibitors malathion and PBO substantially increased the susceptibility of the MHR population (Table 4 and Figure 2A), reducing the GR_{50} values of pyroxsulam by 30% and 60% (*t*-test, $p < 0.05$), respectively. PBO and malathion decreased detoxification in MHR for the ALS-inhibiting pyroxsulam to some extent, but the level of resistance remained high in the resistant population (R/S GR_{50} ratios of 58.25 and 97.52, respectively), suggesting that TSR played a major role in the overall resistance.

3.3. Resistance to PSII-Inhibiting Isoproturon

Apart from the resistance to pyroxsulam and pinoxaden, the single *L. multiflorum* MHR population was demonstrated to have evolved moderate resistance to isoproturon, which is a PSII-inhibiting herbicide. The estimated GR_{50} values for MHR and HS were 307.21 g a.i. ha^{-1} and 42.15 g a.i. ha^{-1}, respectively (Table 4 and Figure 3A). According to the RI values, the MHR population presented 7.29-fold moderate resistance to isoproturon.

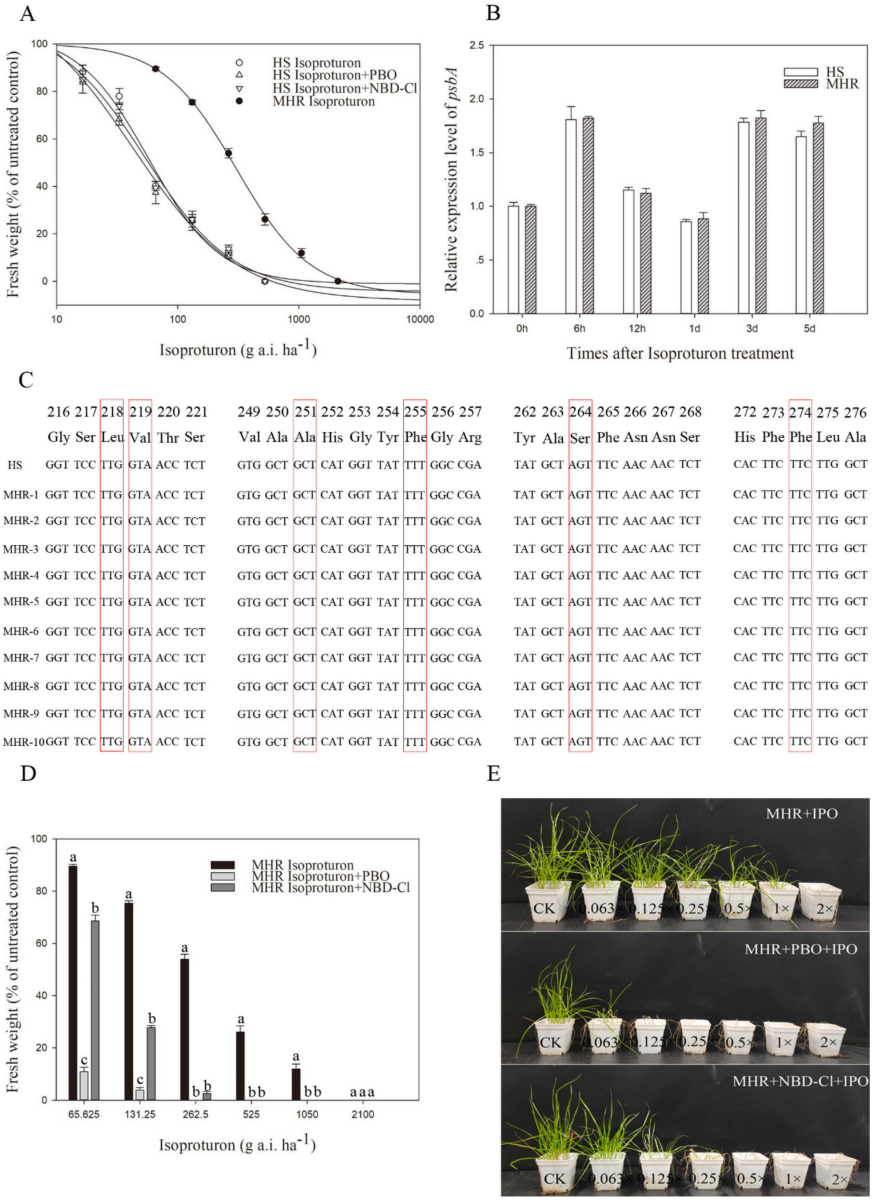

Figure 3. The MHR population has evolved resistance to PSII-inhibiting isoproturon. (**A**) Percentage fresh weight with isoproturon and/or P450 inhibitor treatment in the whole-plant dose-response assay. (**B**) *psbA* gene expression analysis. (**C**) Nucleotide sequence of the *psbA* gene in HS and MHR *L. multiflorum* biotypes. (**D**) Biomass difference in MHR populations treated with isoproturon in the absence and presence of CYP450 inhibitor PBO and GST inhibitor NBD-Cl. (**E**) Phenotype differences in HS and MHR populations treated with isoproturon (IPO) in the absence and presence of CYP450 inhibitor PBO and GST inhibitor NBD-Cl. Vertical bars represent the mean ± SE. Amino acid sequence positions refer to the sequence of *psbA* from *Arabidopsis thaliana* (GenBank accession number X79898.1). Seven positions endowing resistance to PSII inhibitors are marked with red boxes. Different letters indicate different significance levels (*t*-test, $p < 0.01$).

Nucleotide sequence analysis revealed that no mutations associated with resistance to this PSII inhibitor were found in the MHR population, suggesting the moderate isoproturon resistance was driven by other potential mechanisms (Figure 3C).

Changes in *psbA* gene expression level were measured at six time points (0, 6 h, 12 h, 1 d, 3 d, 5 d) after isoproturon treatment in both populations using the relative quantification method. There was no significant difference (*t*-test, $p > 0.05$) in expression between the resistant and sensitive populations in different treatments (Figure 3B). The *psbA* gene expression in both populations showed the same trend after 6 h, 12 h and 1 d of isoproturon treatment, reaching a peak value after 6 h of isoproturon treatment and then a minimum at 1 d. Interestingly, the expression of HS and MHR started to increase again after 3 d of isoproturon treatment and decreased at 5 d.

However, CYP450- and GST-mediated NTSRs were confirmed in the isoproturon-resistant biotype. Both inhibitors at their respective doses were observed to show no discernible influence on *L. multiflorum* plants. Treatment with PBO or NBD-Cl plus isoproturon had no substantial effect on the GR_{50} values of HS (Table 4) but increased isoproturon toxicity to MHR (Figure 3E), with fresh weight decreasing from 89% to 10% and 68% at the lowest dose, respectively (Figure 3D). In particular, compared to NBD-Cl, the addition of PBO increased the biotoxicity of isoproturon to MHR by 57% and 23% at doses of 65.63 g a.i. ha^{-1} and 131.25 g a.i. ha^{-1}, respectively, indicating a stronger potentiation effect of PBO on isoproturon (Figure 3D).

3.4. Sensitivity of L. multiflorum to Other Herbicides

Other than the resistance to pyroxsulam, pinoxaden and isoproturon, this single MHR *L. multiflorum* biotype also displayed different resistance levels to 10 other ALS- or ACCase-inhibiting herbicides. The GR_{50} values of MHR and HS populations for several tested herbicides are shown in Table 4. Based on the RI values, MHR exhibited 12.00-, 12.16-, 13.02-, 34.61-, 42.22- and 230.20-fold resistance to the ALS inhibitors bispyribac-sodium, imazethapyr, imazamox, penoxsulam, mesosulfuron-methyl and flucarbazone-sodium, respectively. For the ACCase inhibitors, MHR exhibited 30.77-, 44.12-, 101.34- and 152.67- fold resistance to fenoxaprop-P-ethyl, clodinafop-propargyl, tralkoxydim, and sethoxydim, respectively.

4. Discussion

Resistance to ACCase-inhibiting (group 1 HRAC/WSSA) and/or ALS-inhibiting herbicides (group 2 HRAC/WSSA) in independent *L. multiflorum* populations has been documented worldwide [1]. Several *L. multiflorum* populations from UK were also found to have evolved multiple resistances to the PSII-inhibiting herbicide (group 5 HRAC/WSSA) isoproturon and ACCase inhibitors in 2002 [42]. However, after that, there were no records of the resistance of *L. multiflorum* to isoproturon. Except for the confirmed field-evolved resistance to herbicides from Group 1 and Group 2, the tested *L. multiflorum* population from wheat fields in China in this study has evolved multiple resistance to isoproturon from Group 5. This is the first report of a single *L. multiflorum* population evolving resistance to PSII inhibitors in addition to ALS and ACCase inhibitors in China.

Weeds evolve diverse resistance mechanisms to overcome the selection pressure caused by long-term use of herbicides, and TSR was reported in a range of resistance cases. *ACCase* gene sequencing analysis revealed a frequent mutation Ile-1781-Leu and a rare mutation Ile-2041-Val in the MHR population. It was noted that two different nucleotide substitutions (ATA to CTA and ATA to TTA) resulted from the amino acid replacement of Ile to Leu in the Ile-1781 allele and individuals with the corresponding genotypes survived pinoxaden application relative to HS population. Mutations in Ile-1781 allele were characterized in numerous weeds and could endow broad resistance spectrums to APPs, CHDs and PPZ [43]. Given the cross-pollination characteristics of *L. multiflorum*, multiple resistance alleles at the 1781 position will accumulate via pollen-mediated gene flow [44] in various biotypes if no effective approaches are applied.

In contrast with the more prevalent Ile-1781-Leu mutation in weed species, including *L.multiflorum*, the Ile-2041-Val mutation was less frequent and had only been documented in *Lolium rigidum* Gaud. [45,46] and *Beckmannia syzigachne* Steud. [47]. Although the Ile-2041-Val mutation was identified in both weed species, the evolutionary roles of field-resistance and the cross-resistance patterns conferred were quite different. In *B. syzigachne*, Wang et al. [47] found that the single Ile-2041-Val mutation could confer resistance to certain ACCase-inhibiting APP, CHD and PPZ herbicides. However, the Ile-2041-Val mutant population in *L. rigidum* presented dominant or partially dominant resistance to APP herbicides and a moderate resistance to PPZ herbicide pinoxaden, but not to CHD herbicides [45]. Moreover, in both weed species, different resistance phenotypes were observed in resistant populations carrying various 2041 ACCase genotypes. For example, in *L. rigidum*, heterozygous individuals carrying only one 2041-Val *ACCase* allele presented more herbicide tolerance and survived at a slightly-higher-than-recommended dose of pinoxaden relative to homozygous individuals. As a homologous species of *L. rigidum*, the tested MHR population of *L. multiflorum* with the same mutation in the present study whether presented the similar pattern: accumulation of the 2041-Val *ACCase* mutant allele results in more adaption penalty rather than enhanced tolerance to herbicides. Further investigation isolating subpopulations with different mutation alleles are required to evaluate the accuracy of hypothesis and uncover the hidden herbicide resistance profiles for resistance management of *L. multiflorum*.

Different ALS mutations conferring TSR have been identified in numerous weed species. Among these mutations, the 197 and 574 positions are the most common sites known to cause distinct resistances to different ALS inhibitors [1]. In this study, phenotype resistance to pyroxsulam was observed in the resistant population carrying Pro-197-Thr, Pro-197-Gln or Trp-574-Leu, suggesting that mutations in the *ALS* gene are one of the mechanisms resulting in the high resistance of *L. multiflorum* to pyroxsulam. Multiple resistance mutations may be associated with specific ALS inhibitors and herbicide application histories [14].

In addition to the TSR to pyroxsulam, NTSR mediated by CYP450 was present in this MHR population and may confer resistance in individuals without TSR mutations. Besides, we observed that PBO was proved to be more effective than malathion during the synergistic application of CYP450 inhibitors and pyroxsulam. It has been shown that PBO can be used as an effective synergist to enhance the toxic effect of ACCase [20,48] and PSII [49] inhibitors on weeds. Therefore, combined with the available data, we speculate that this result may be due to the action of PBO on different CYP450 genes involved in the response to the three herbicide MoAs. However, validation of the potentiation effect of PBO involved in ACCase inhibitors is essential.

No target mutation associated with TSR was found in the *psbA* gene, which indicates that additional mechanisms may exist and endow the moderate isoproturon resistance in MHR. In fact, not all resistance cases were responsible for target gene mutations, as observed not only in ALS and ACCase inhibitors, but also in PSII inhibitors [50–52]. In contrast to the observed overexpression of the *psbA* gene in several weeds resistant to PSII inhibitors [6,53], there was no significant difference in expression of the *psbA* gene after isoproturon treatment in the MHR population compared with its counterpart population. However, the phenotype resistance to isoproturon could be reversed by the CYP450 inhibitor PBO and the GST inhibitor NBD-Cl, which indicated that CYP450- and GST-mediated NTSRs may endow isoproturon resistance to the MHR population without target mutation. Metabolic resistance was also recorded in some cases of resistance to PSII inhibitors. Enhanced bioefficacy of isoproturon against *Phalaris minor* was observed after the addition of PBO [49]. Besides several CYP450 genes, resistance to the PSII inhibitor atrazine in *Commelina communis* L. was also associated with GST genes [53]. With the help of RNA-Seq, further detailed investigation will dissect the roles of candidate CYP450 and GST genes in NTSR.

Target gene mutations conferring herbicide resistance may lead to pleiotropic effects on plant fitness [54,55]. In our studies, we did find target gene mutations in ALS and ACCase; we are working on this Italian ryegrass MHR population to test whether they have any fitness penalty.

In conclusion, we found that a single *L. multiflorum* population from Henan Province, China, had evolved multiple herbicide resistance to 13 herbicides from three MoAs, including ALS-inhibiting pyroxsulam, ACCase-inhibiting pinoxaden and PSII-inhibiting isoproturon. In the MHR population, resistance to ACCase and ALS inhibitors involved target gene mutations (Pro-197-Gln, Pro-197-Thr, and Trp-574-Leu in *ALS*, Ile-1781-Leu and Ile-2041-Val in *ACCase*). Meanwhile, CYP450- and/or GST-mediated NTSRs accounted for the partial pyroxsulam resistance and moderate isoproturon resistance and were likely involved in the evolution of resistance to multiple herbicides. All our results indicate that *L. multiflorum* was at a high risk of resistance evolution in wheat fields in China and that integrated weed management approaches are increasingly required to mitigate the spread of resistance.

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