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The molecular basis of florasulam resistance and differential sensitivity to alternative herbicides in cleavers

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Research Article

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Keywords:
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Abstract

Cleavers, an annual or winter annual broadleaf weed in the Rubiaceae family, has become troublesome in the wheat fields of the Huang-Huai-Hai region in China due to its herbicide resistance. In North America the common name of the plant is stickwilly; in China it known as cleavers. Four populations of cleavers (JS-15, SD-10, JS-22, and AH-20) were collected from wheat fields in Jiangsu, Shandong, and Anhui provinces, where the plant was not being controlled with applications of florasulam. The aims of this study were to identify the herbicide resistance patterns and investigate the mechanism underlying florasulam resistance. Whole-plant dose-response experiments revealed a notable variation in the degree of resistance exhibited by three specific populations toward florasulam, in comparison to the most sensitive population (S and AH-9), with the highest resistance index reaching 841.4. A gene-sequencing assay for acetolactate synthase (ALS) found that plants that were resistant to ALS from the JS-15, JS-22, and AH-20 populations had a Trp-574-Leu mutation, while no known ALS resistance mutations were discovered in SD-10 plants. In vitro ALS enzyme activity assays also indicated that the extractable ALS from JS-15, JS-22, and AH-20 plants was greatly resistant to florasulam relative to plants that are susceptible. Additionally, according to the resistance rating system, all resistant populations were susceptible to carfentrazone-ethyl + MCPA-sodium and bipyrazone + fluroxypyr-methyl. AH-20, JS-15, and JS-22 exhibited resistance to selected ALS, 4-hydroxyphenylpyruvate dioxygenase (HPPD), and photosystem II (PS II) complex inhibitors, demonstrating RR and RRR resistance profiles, whereas AH-9 displayed sensitivity to virtually all tested agents. The SD-10 population, on the other hand, exhibited RR and RRR resistance to HPPD and PS II inhibitors, and sensitivity to tribenuron-methyl. These findings indicate that a target site–based mechanism drives resistance to the ALS inhibitor florasulam in populations of cleavers, but nontarget site resistance may also have contributed to resistance, but this was not investigated. Other herbicides with different sites of action were tested and were active against cleavers.

Introduction

Weed infestation is one of the most significant factors negatively affecting crop production and quality worldwide. The development of herbicide resistance by weeds constrains the sustainable development of weed management strategies, and poses a serious threat to crop production. Cleavers (the common name in North America is stickwilly) is a globally notorious weed belonging to the family Rubiaceae. It is an annual or biennial broadleaf weed with strong adaptability, competitiveness, and reproductive capacity. Cleavers is hermaphroditic and often self-pollinating. Although the anthers may be dry, they still carry pollen and automatically self-pollinate; moreover, four levels of ploidy have been found in this weed (Malik and Born 1988; Taylor 1999). Its seeds are densely covered with hooked bristles, which animals can spread over long distances. The plant has many branches and it sprawls, which can cause crops to lodge and lead to severe yield losses (Bauer et al. 2011; Taylor 1999). Cleavers is widely distributed in China (Wang et al. 2016) and is one of the most harmful weeds in wheat and rapeseed fields in the middle and lower reaches of the Yellow and Yangtze river valleys.

Florasulam, which is applied following an application of tribenuron-methyl, is an important herbicide for the selective control of broadleaf weeds such as cleavers in wheat fields in China. Although florasulam exhibits higher activity and is more stable at low temperatures than tribenuron-methyl, it still belongs to the group of acetolactate synthase (ALS) inhibitors, which is prone to resistance development (Guo et al. 2015; Liu et al. 2015; Ştef et al. 2020). This herbicide is beginning to lose its efficacy against cleavers and many other broadleaf weeds after

several years of continuous use in wheat fields. Herbicide-resistant cleavers has become an urgent problem to be addressed in wheat production in China.

At present, there are two general types of resistance mechanisms in weeds: nontarget-site resistance (NTSR) and target-site resistance (TSR) (Powles and Yu 2010; Qin et al. 2022). The NTSR mechanism of weeds is complex and usually controlled by multiple genes, leading to unpredictable patterns of cross-resistance. For example, the most frequently reported NTSR mechanism often involves enhanced metabolism through increased activity of cytochrome P450 monooxygenases, glutathione S-transferases, and glycosyl transferases (Délye et al. 2007; Yu and Powles 2014a). In many instances, TSR occurs due to mutations in target genes, which reduce enzyme sensitivity to herbicides (Powles and Yu 2010). Among the top globally reported cases of ALS inhibitor resistance, eight resistance mutation sites located in five conserved domains have been proven to confer herbicide resistance in weeds, and at least 29 amino acid substitutions have been identified in 174 ALS inhibitor-resistant weed species so far (Heap 2024; Tranel et al. 2024).

Herbicide resistance to tribenuron-methyl by cleavers in China was first reported by Peng et al. (2009). Since then, more populations have been documented to have evolved resistance to tribenuron-methyl. A comprehensive study of the resistance exhibited by cleavers toward tribenuron-methyl, bensulfuron-methyl, and halosulfuron-methyl highlighted the significance of site diversity (Lou et al. 2022). Florasulam was introduced in 2011 for use against weeds in wheat fields in China after the failure of tribenuron-methyl to control cleavers and other broadleaf weed species. Unfortunately, many farmers from the main wheat production areas have noted that the efficacy of florasulam (and even in a mixture with other herbicides) has been declining in recent years. The present study aimed to investigate the mechanism underlying florasulam resistance in cleavers populations and to identify their sensitivity to several other herbicides and herbicide mixtures, with a goal of maintaining the sustainable control of this weed.

Materials and Methods

Plant Materials

In this study, five populations of cleavers were used. One population, AH-9, was confirmed to be sensitive to florasulam in a preliminary screening test and was used as the S, or susceptible, population. The seeds of four cleavers populations that were suspected to be resistant to florasulam were collected from wheat fields with a history of florasulam application of at least 4 yr in Shandong, Jiangsu, and Anhui provinces. In those regions, growers found that florasulam failed to control cleavers at its maximum field-recommended label rate of 4.5 g ai ha^{-1}. Mature seeds from each population were randomly harvested from individual plants with the infestation area of approximately 0.2 ha. After drying, the seeds were stored at 4 C in paper bags until use. Detailed information about these populations is shown in Table 1.

Whole-Plant Dose-Response Experiment

To promote germination, around 100 capsules containing mature seeds were placed in Petri dishes containing two layers of filter paper (Whatman No. 1, Maidstone, UK) and 6 mL of deionized water (pH = 6.7). The Petri dishes were then placed in a growth chamber (15/10 C, 12/12 h day/night) for germination. After germination, 10 seedlings (after being selected for uniformity) were transplanted into a plastic pot containing loam. The undressed field soil organic matter content of 1.7% was filtered using a 3-mm sieve. The plants were grown in a greenhouse in November with natural light at a temperature of 25/20 C day/night. The pots were watered every 2 d to maintain moisture.

When the seedlings reached the 3-leaf stage and plant height was 5 to 15 cm, they were treated with herbicides and then returned to the greenhouse. Based on a preliminary experiment, the doses of florasulam applied to cleavers populations (JS-22、SD-10、AH-20、JS-15) were 0, 0.9, 4.5, 22.5, 112.5, 562.5, and 2,812.5 g ai ha^{-1}; and 0, 0.036, 0.18, 0.9, 4.5, 22.5 and 112.5 g ai ha^{-1} was applied to S (susceptible) population AH-9. Herbicides were applied using a mobile nozzle cabinet sprayer equipped with flat-fan nozzles (TeeJet 9503EVS; Greenman Machinery, Beijing, China) that delivered a spraying volume of 450 L ha^{-1} at 280 KPa. After 21 d, all aboveground shoots were collected, and fresh weight was recorded for each treatment. The experiment was conducted as a completely randomized design, and was repeated three times.

ALS Gene Sequencing Assay

Fresh leaf tissue from each population at the 3-leaf stage was harvested and stored at −80 C. To ensure that the resistant plants were selected for gene sequencing, the plants of each resistant population that survived treatment with 4.5 g ai ha^{-1} florasulam were used in the following experiment. Approximately 50 mg of fresh leaves (mixed samples of cleavers plants) were used to extract genomic DNA using an EasyPure® Plant Genomic DNA Kit (TranGen Biotech, Beijing, China). The concentration of the extracted DNA was approximately 120 ng μl^{-1} and the ratio of A260/280 was approximately 2.0, demonstrating its purity. Twenty individual plants were selected from each population. One primer pair (forward: 5′-ACAGCATCAC CGCCTTGTT-3′; 5′-TC CCATCTCC CTCGGTAATC-3′) was used to amplify the *ALS* genes containing the eight known resistance mutation sites. The polymerase chain reaction (PCR) mixture contained 17 µL of double-distilled water (Coolaber, Beijing, China), 2 µL of each primer (10 µM, Sangon Biotech, Shanghai, China), 4 µL of genomic DNA, and 25 µL of 2×Phanta Max Master Mix (Dye Plus; Vazyme, Nanjing, China) in a final volume of 50 µL. The PCR conditions were as follows: denaturation at 95 C for 30 s, 34 cycles at 95 C for 30 s, 52 C for 15 s and 72 C for 80 s, with a final step of 72 C for 5 min.

The PCR products were detected using 1.0% agarose gels and purified using an EasyPure Quick Gel Extraction Kit (TransGen Biotech). The purified PCR products were cloned with the pEASY®-T1 Cloning Kit (TransGen Biotech) and transferred into Trans1-T1 Phage-Resistant Chemically Competent Cells (TransGen Biotech). At least 10 positive clones from each individual plant were sent to the Sangon Biotech company (Shanghai, China) for sequencing. The *ALS* gene of cleavers was aligned and compared using the DNAMAN software package (v. 6.0.3.99; Lynnon Biosoft, QC, Canada).

In vitro ALS Activity Assay

The fresh leaf tissue from the resistant and susceptible populations was harvested at the 3-leaf stage. The ALS extraction and herbicide inhibition assays were performed in vitro, according to methods described by Yu et al. (2004) and Bradford (1976). The concentration of acetoin was determined via colorimetry to be 530 nm with a UV spectrophotometer (Epoch; BioTek Instruments, Winooski, VT) to measure the ALS activity. Three subsamples from each extraction

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Table 1. Collection information and resistance status of cleavers populations.

Population	Status[a]	Location			Longitude & Latitude	Site	Number of florasulam applications
		Village	County	Province			
JS-22	R	Gugao	Taizhou	Jiangsu	119.928996°E, 32.462137°N	Wheat field	≥ 5
SD-10	R	Renping	Liaocheng	Shandong	116.26117°E, 36.587482°N	Wheat field	≥ 5
AH-20	R	Mengcheng	Bozhou	Anhui	116.571588°E, 33.271658°N	Wheat field	≥ 5
JS-15	R	Fuan	Dongtai	Jiangsu	120.489732°E, 32.679501°N	Wheat field	≥ 5
AH-9	S	Yingzhou	Fuyang	Anhui	115.818134°E, 32.89703°N	Uncultivated land	Never applied

[a]Abbreviations: R, resistant; S, susceptible.

Table 2. Herbicides used in cross-resistance identification and their application rates.

Site of action[a]	HRAC group[b]	Herbicide[c]	Recommended label rate	Resistance profile[d]				
				SD-10	AH-20	AH-9	JS-15	JS-22
			g ai ha^{-1}					
ALS	2	Pyroxsulam	15	RR	RR	S	RRR	RRR
ALS	2	Tribenuron-methyl	22.5	S	RRR	R?	RRR	RR
AM	4	Fluroxypyr	210	S	S	S	S	S
AM	4	MCPA-sodium	1260	S	S	S	S	S
PPO	14	Carfentrazone-ethyl	30	S	S	S	S	S
HPPD	27	Bipyrazone	37.5	RRR	RRR	S	RRR	RR
HPPD	27	Cypyrafluone	180	RR	RR	S	RR	RR
AM+HPPD	4+27	Fluroxypyr+ bipyrazone	476	S	S	S	S	S
PPO+AM	14+4	Carfentrazone-ethyl+MCPA-sodium	165	S	S	S	S	S
PS II	5	Isoproturon	1,200	R?	RR	S	RR	R?

[a]SOA represents for site of action. SOA inhibitors include the following: ALS, acetolactate synthase; AM, auxin mimics; HPPD, 4-hydroxphenylpyruvate dioxygenase; PPO, protoporphyrinogen oxidase; PS II, photosystem II.
[b]Herbicide groups as categorized by the Herbicide Resistance Action Committee (HRAC).
[c]Pyroxsulam (4% OD; Dow Agrosciences, America), tribenuron-methyl (10% WP; Greenland, Shandong, China), fluroxypyr (200 g L^{-1} EC; Luba Chemical, Shandong, China), MCPA-sodium (56% SP; Greenland), carfentrazone-ethyl (10% WP; Ruidong, Jiangsu, China), bipyrazone (10% OD; Kingagroot, Jiangsu, China), cypyrafluone (6% OD; Kingagroot), isoproturon (50% WP; Kuida Agrochemical, Jiangsu, China), fluroxypyr + bipyrazone (22% OD; Kingagroot), carfentrazone-ethyl+MCPA-sodium (70.5% WG; Fumeishi, Jiangsu, China; 4% carfentrazone-ethyl + 66.5% MCPA-sodium).
[d]Susceptibility was classified according to the "R" resistance rating system described by Moss et al. (2007) where RRR and RR indicate confirmed resistance, highly and probably reducing herbicide performance; respectively; R? indicates resistance may be evolving, possibly reducing herbicide performance; and S indicates susceptible.

were assayed, and there were three extractions for each population. The florasulam (97%, Shandong Greenland Chemical Co., Jinan, China) concentrations used for the enzyme inhibition assays were 0.0001, 0.001, 0.01, 0.1, 1, 10, 100, and 1,000 µM for the resistant and susceptible populations.

Sensitivity to Other Herbicides with Various Sites of Action

The present study also aimed to determine the sensitivity of all populations to 10 other herbicides with different sites of action (Table 2). The plants were cultivated to the 3-leaf stage and treated with the florasulam recommended field dose of 4.5 g ai ha^{-1}. After 21 d, the shoots of the aboveground portion were dried, and the fresh weight was recorded. All treatments were replicated three times, and the experiment was conducted three times. The herbicide sensitivity of the resistant and susceptible populations was were divided into Moss (2007) four resistance categories (RRR, RR, R?, and S) as described elsewhere, based on the percentage of biomass reduction. RRR marks the "crisis stage" of weed resistance, with the highest levels of resistance; RR represents a high degree of resistance, intermediate stage of resistance evolution, and timely intervention can avoid escalation to RRR; R? indicates that the resistance of the weed to a specific herbicide has not been fully confirmed. Resistance may be suspected, but further research is needed for verification. S indicates that the weed is susceptible to the herbicide, meaning the herbicide can effectively control this weed.

Statistical Analyses

The plant or ALS data were subjected to one-way ANOVA (all of the parameters were significant at $P \leq 0.05$). The data were pooled before analysis when no significant differences between the repeated experiments were observed in the ANOVA. The data were subjected to a nonlinear regression analysis using SigmaPlot 14 (Systat Software, San Jose, CA). The herbicide rate or herbicide dose resulting in 50% growth reduction (GR_{50}) or 50% inhibition of ALS activity (I_{50}) was estimated using the four-parameter log-logistic model as follows:

$$y = C + \{(D - C) / [1 + (x/GR_{50})^b]\} \quad [1]$$

where C is the lower limit, D is the upper limit, b is the slope, and GR_{50} is the effective dose/concentration causing 50% inhibition/reduction. The resistance index (RI) was calculated by dividing the determined GR_{50} value of the resistant population by that of the sensitive population (Zhang et al., 2022).

Results and Discussion

Susceptibility of Populations of Cleavers to Florasulam

The susceptibility of the resistant and susceptible populations to florasulam was evaluated by using whole-plant dose-response experiments (Table 3, Figure 1). The florasulam sensitivity of all

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Table 3. GR_{50} values of susceptible and resistant populations of cleavers in response to florasulam.[a,b]

Population	C	D	B	GR_{50}	RI
	g ai ha^{-1}	g ai ha^{-1}		g ai ha^{-1}	
SD-10	−3.4 (28.1)[f]	97.8 (34.1)	−0.7 (0.4)	2.0 (1.9)	10.7
JS-22	17.9 (2.4)	79.5 (9.4)	−1.0 (0.2)	71.6 (20.6)	376.8
JS-15	13.0 (2.3)	84.6 (18.9)	−1.4 (0.6)	159.9 (69.7)	841.4
AH-20	18.2 (1.5)	56.5 (3.2)	−2.61 (0.83)	73.90 (11.62)	388.9
AH-9	−74.2 (129.7)	174.5 (134.3)	−0.5 (0.2)	0.2 (0.5)	–

[a]Abbreviations: C, lower limit; D, upper limit; B, slope of curve in GR_{50}; GR_{50}, the herbicide dose or concentration resulting in 50% growth inhibition; RI, resistance index, a value calculated as the ratio of GR_{50} value of resistant populations and susceptible population (AH-9).
[b]Each data point represents the mean ± SEM of three replicate treatments.

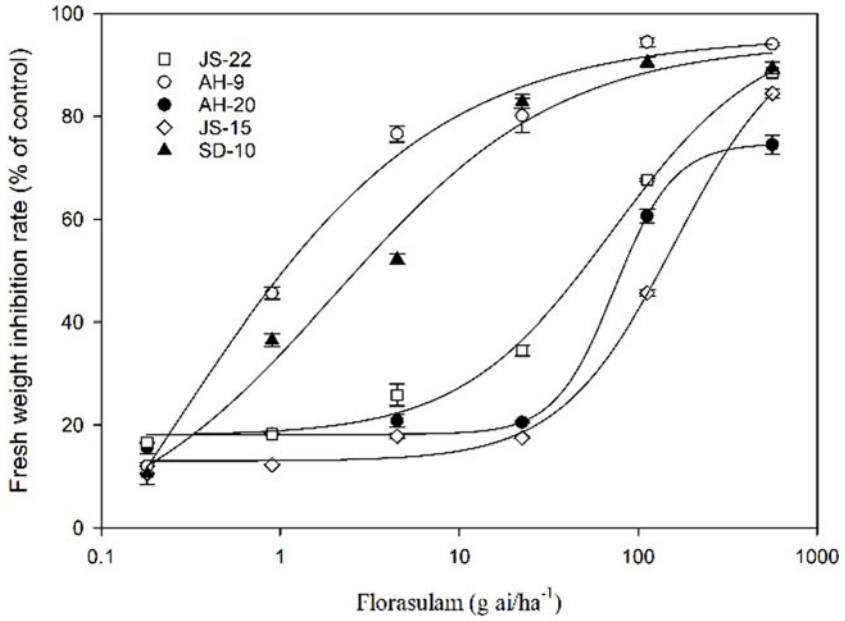

Figure 1. Dose–response curves for fresh weight inhibition (percent of control) of the resistant and susceptible (AH-9) populations with florasulam treatment. The recommended field-use dosage is 4.5 g ai ha^{-1}. Each data point represents the mean ± SEM of three replicate treatments.

putative resistant populations was obviously reduced compared with the susceptible population AH-9. The GR_{50} values of florasulam for the putative resistant populations AH-20, JS-15, JS-22, and SD-10, and the AH-9 susceptible population were 73.9, 159, 71.6, 2.04 and 0.19 g ai ha^{-1}, respectively (Table 3). In particular, the GR_{50} values for AH-20, JS-15, and JS-22 were much higher than the recommended label field rate of 4.5 g ai ha^{-1}. As a result, all tested resistant populations evolved resistance to florasulam, with RIs ranging from 10.74-fold to 841.37-fold.

ALS Gene Sequencing and Sequences Analysis

In this study, 10 plants from each population were randomly selected and bulked for *ALS* gene sequencing. An *ALS* gene fragment of 1,908 base pairs, including the five known conserved domains, was amplified from each population. The sequences from the different populations showed 94.35% identity with the documented cleavers *ALS* gene (GenBank accession GU377313.1), indicating that the target genes were amplified. Moreover, all sequenced *ALS* genes from the resistant and susceptible populations showed 93.7% similarity. The majority of the single-nucleotide polymorphisms were synonymous without causing amino acid substitution.

The AH-9 plants did not exhibit ALS inhibitor resistance that would endow mutations. Conversely, in the resistant AH-20, JS-15, and JS-22 populations, the well-known resistance mutation Trp-574-Leu was identified in the *ALS* gene. Furthermore, when comparing the *ALS* gene sequences between the resistant and susceptible populations, no other known amino acid mutations were found in the five known conserved domains. In addition, no known ALS resistance mutations were detected in the SD-10 population. The resistance mechanisms underlying this specific population are worthy of further investigation. In the following transcriptome sequencing and gene expression profiling of selected resistant and susceptible populations, there was no observed difference in *ALS* gene expression (unpublished data). In addition, no known ALS resistance mutations were detected in the SD-10 population, indicating that the overexpression of the target gene and/or NTSR mechanisms may be driving the florasulam resistance. In our subsequent analysis, which encompassed transcriptome sequencing and expression profiling of select populations, we observed no discernible shift in the *ALS* gene expression pattern in the resistant populations, in which no mutation in the targeted gene was detected, in comparison to the susceptible populations.

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Table 4. Nucleotide sequence alignment of acetolactate synthase and derived amino acids of resistant and susceptible cleavers.[a]

Population	Amino acid position and relative sequence of nucleotide and amino acid								
	122	197	205	206	376	377	574	653	654
Arabidopsis tdaliana	GCG	CCT	GCT	TTC	GAT	CGT	TGG	AGT	GGC
JS-22	GCG	CCT	GCT	TTC	GAT	CGT	**TTG**[b]	AGT	GGC
AH-20	GCG	CCT	GCT	TTC	GAT	CGT	**TTG**[b]	AGT	GGC
JS-15	GCG	CCT	GCT	TTC	GAT	CGT	**TTG**[b]	AGT	GGC
AH-9	GCG	CCT	GCT	TTC	GAT	CGT	**TGG**[b]	AGT	GGC
SD-10	GCG	CCT	GCT	TTC	GAT	CGT	TGG	AGT	GGC

[a]Nucleotide sequence abbreviations: AGT (S_{653}), serine; CCT (P_{197}), proline; CGC (G_{654}), glycine; CGT (R_{377}), arginine; GAT (D_{376}), aspartic acid; GCG (A_{122}), alanine; GCT (A_{205}), alanine; TTC (F_{206}), phenylalanine; TGG (W_{574}), tryptophan; TTG (L_{574}), leucine.
[b]Mutated nuclides and mutated amino acid residues.

Table 5. Total ALS activity and I_{50} determined with a partially purified ALS enzyme isolated from susceptible and resistant populations of cleavers.[a,b]

Population	S/R	Total ALS activity		I_{50}		RI[c]
		nmol acetoin mg^{-1} protein min^{-1}		μM		
AH-9	S	17.54 ± (0.20)	d	0.02 ± (0.01)	d	1.00
JS-22	R	20.16 ± (0.15)	b	62.05 ± (7.19)	b	3,165.91
AH-20	R	18.16 ± (0.21)	c	74.10 ± (3.81)	a	3,780.70
SD-10	R	17.44 ± (0.44)	d	0.03 ± (0.02)	d	1.58
JS-15	R	26.36 ± (0.13)	a	34.53 ± (5.19)	c	1,762.21

[a]Abbreviations: ALS, acetolactate synthase; I50, 50% inhibition of ALS activity; S/R, susceptible/resistant; RI, resistance index.
[b]Values represent the mean (±SEM) of two experiments, each containing three replicates. Values followed by different letters within the same column are significantly different ($P = 0.05$).
[c]RI was calculated by dividing the I_{50} value of the resistant population by that of the susceptible population.

In vitro ALS Inhibition Assay

To assess the differential sensitivity of the ALS enzyme within the resistant and susceptible populations of cleavers, inhibition of in vitro ALS activity was measured both with and without florasulam. The total extracted ALS activity showed no notable difference between the resistant and susceptible populations (Table 4). In the presence of florasulam, the enzyme activity of the susceptible population AH-9 and the resistant population SD-10 was inhibited, with I_{50} values of 0.0196 μM and 0.031 μM, respectively. Conversely, the sensitivity of the ALS extracted from AH-20, JS-15, and JS-22 plants was significantly inhibited, with I_{50} values of 74.1, 34.5, and 62.1 μM, respectively, which were 3,780.7, 1,762.2, and 3,165.9 times higher than that of the wild-type ALS (Table 5, Figure 2).

Cross Resistance to Other Herbicides

To determine the sensitivity of cleavers to other herbicides and herbicide mixtures, we carried out a single-dose resistance test using the field use rate listed on the product label as the discriminating dose. Two herbicides that inhibit ALS (categorized as Group 2 herbicides by the Herbicide Resistance Action Network [HRAC] and the Weed Science Society of America), one that inhibits PS II (a Group 5 herbicide), two that inhibit auxin mimics (Group 4), one that inhibits protoporphyrinogen oxidase (PPO; Group 14) (Wang et al. 2020), and two herbicides from Group 27 that inhibit HPPD (Heap 2024) and their mixtures were tested, as listed in Table 2. Based on the resistance classification system, the JS-22 population exhibited RRR and RR profiles to the herbicides pyroxsulam (Group 2), tribenuron-methyl (Group 2), bipyrazone (Group 27), and cypyrafluone (Group 27), and a R? profile to isoproturon (Group 5).

Nonetheless, the JS-22 population remained susceptible to fluroxypyr (Group 4), MCPA-sodium (Group 4), and carfentrazone-ethyl (Group 14) herbicides. These results indicated that the JS-22 population had evolved resistance to pyroxsulam and tribenuron-methyl, both of which are ALS inhibitors (Table 2). The herbicide sensitivity profiles of the other resistant populations were similar to those of JS-22. Cypyrafluone and bipyrazone are new herbicides categorized as HPPD inhibitors. They are used to control annual broadleaf weeds in winter wheat fields. Greenhouse experiments to determine the control efficacy against cleavers ranged from 7.5% to 65.1% when cypyrafluone was applied, and from 8.0% to 80.2% when bipyrazone was applied. Interestingly, the ability of these herbicides to control resistant populations of cleavers varied among samples from various regions. There were large fluctuations in weed control effectiveness, indicating an unsatisfactory level of control. All populations demonstrated sensitivity to the combined application of fluroxypyr + bipyrazone and carfentrazone-ethyl + MCPA-sodium mixtures, providing a valuable reference for the effective control of cleavers through the utilization of chemical combinations that have different sites of action. We hypothesize that these resistant populations may also exhibit cross-resistance to numerous other herbicides, including isoproturon, a PS II inhibitor. It is crucial to focus on developing integrated strategies that delay the development of resistance and promote the sustainable control of this challenging weed in wheat fields.

In this research, 10 different types of herbicides were chosen to identify their efficacy against resistant populations of cleavers. The resistant populations developed cross-resistance to many herbicides, particularly ALS inhibitors. All four resistant populations that were tested showed less sensitivity to the PS II inhibitor isoproturon. Moreover, the sensitivity of these resistant populations to two new HPPD-inhibiting herbicides, cypyrafluone and bipyrazone, varied. Fortunately, MCPA-sodium, fluroxypyr, carfentrazone-ethyl, and their mixtures provided high levels of control among resistant populations, exceeding 90% to 100% fresh weight inhibition. These screened herbicides and their mixtures will provide alternatives for the control of cleavers and an assessment of their efficacy in a field environment is needed.

The ALS inhibitor tribenuron-methyl and the auxin mimic herbicide MCPA-sodium were introduced in 1988 and 1952, respectively, and became the standards for efforts to control cleavers. However, an overreliance on chemical control and single

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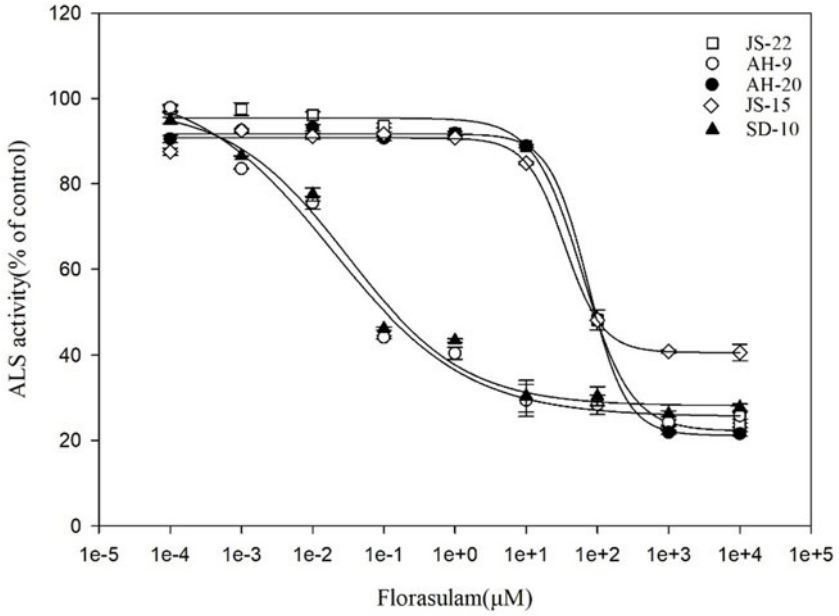

Figure 2. Inhibition assay of acetolactate synthase isolated from resistant and susceptible cleavers by florasulam.

use of these herbicides led to the rapid evolution of resistance by cleavers. At present, florasulam and fluroxypyr serve as the main herbicides for the control of cleavers in winter wheat fields in China. Although florasulam offers advantages over tribenuron-methyl, it belongs to a group of ALS inhibitors that have a high risk of resistance evolution, and the continuous use of ALS inhibitors for more than 3 to 5 yr can lead to the development of weed resistance (Yu and Powles 2014b; Zhang et al. 2009). In this study, resistant cleavers populations were collected from wheat fields with a long history (more than 15 to 20 yr) of ALS inhibitor use. Anecdotal reports have found that many populations of cleavers have developed resistance to tribenuron-methyl. This study documents the first florasulam resistance by cleavers confirmed in China. Cleavers has been shown to develop resistance toward the herbicides tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl (Lou et al. 2022). Concurrently, false cleavers (Galium spurium), a species belonging to the same taxonomic family has exhibited resilience against florasulam and underwent mutations at varying loci (Beckie et al. 2012). The resistant populations tested in this study were collected from wheat fields that had relied extensively on herbicides that included tribenuron-methyl and florasulam, as well as other herbicides. Numerous studies have noted that the evolution of the resistance to ALS inhibitors (e.g., florasulam) (Tranel et al 2024) is related to the high herbicide selection pressure due to its application history (Tranel and Wright 2002). In China, Bi et al. (2016) observed varied resistance levels to fenoxaprop-P-ethyl and mesosulfuron-methyl in a population of *Alopecurus japonicus* (foxtail), which is attributed to the substitution of Ile-1781 in ACCase with Leu and Trp-574 in *ALS* with Leu, respectively. Guo et al. (2016) reported similar resistance to both fenoxaprop-P-ethyl and mesosulfuron-methyl in an *A. aequalis* population, stemming from mutations in Ile-2041 of ACCase and Pro-197 to Arg in *ALS*, respectively.

Resistance endowed by one or more mutations in the *ALS* gene is the most frequently identified mechanism associated with ALS inhibitor resistance, and the resistance mutations are diverse among different weed species (Yu and Powles 2014b). The *ALS* gene sequencing analysis verified that a Trp-574-Leu mutation (Zhao et al. 2020) exists in cleavers plants that are resistant to florasulam, as reported in a number of weed species that have developed resistance to ALS-inhibiting herbicides, indicating that the Trp-574-Leu mutation represents the molecular basis of TSR in florasulam-resistant cleavers in these populations.

We also found that florasulam resistance by cleavers has become a serious problem in many major wheat production areas, including Shandong, Henan, Jiangsu, Anhui, and other provinces. More than 54% of the populations we tested have evolved resistance to florasulam, with some of them even exhibiting resistance to MCPA-sodium (unpublished data). In this study, some highly resistant populations were reported to possess amino acid mutations, which may be the reason for their high level of resistance. In many cases, resistance to ALS inhibitors typically arises from single-point mutations that lead to amino acid substitutions at the enzyme's herbicide-binding site, diminishing the herbicide sensitivity (Délye 2013; Powles and Yu 2010). The Trp-574 residue is particularly critical, changing the shape of the active-site channel and interfering with the binding of ALS-inhibiting herbicides to the enzyme (Duggleby et al. 2008). Our research revealed that the ALS from the AH-20, JS-15, and JS-22 populations exhibited a marked decrease in florasulam sensitivity, which correlates with the observed high levels of resistance. These findings support previous reports of resistance by various species such as shepherd's purse (*Capsella bursa-pastoris* (L.) Medik.) resistance to tribenuron-methyl (Zhang et al. 2017), flixweed (*Descurainia sophia* L.) resistance to ALS inhibitors (Yang et al. 2016), corn gromwell (*Lithospermum arvense*) resistance to tribenuron-methyl (Wang et al. 2019), and shortawn foxtail (*Alopecurus aequalis* Sobol.) resistance to mesosulfuron-methyl (Zhao et al., 2018).

Variations in mutations in the *ALS* gene can lead to distinct patterns of cross-resistance among various herbicides. Typically, Pro-197 mutations are linked to resistance against sulfonylureas and triazolopyrimidines; mutations at Ala-122, Ser-653, and Gly-654 are commonly associated with resistance to imidazolidinones; and mutations at Asp-376 and Trp-574 are known to confer wide-ranging resistance across all five structural subgroups of

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ALS-inhibiting herbicides (Délye et al. 2007). Furthermore, Yu and Powles (2014a) highlighted that the pattern of cross-resistance resulting from a site-specific mutation is influenced by the position of the mutation, the particular amino acid changes, the type of ALS inhibitor involved, and occasionally, the species of weed in question. In the present study, the resistant population of cleavers showed notably high resistance to a spectrum of ALS inhibitors, including tribenuron-methyl, florasulam, and pyroxsulam (Yu et al. 2010). This pattern of cross-resistance is consistent with the presence of the Trp-574 mutation, as documented in other resistant grass and broadleaf species (Heap 2024; Tranel et al. 2024). Although TSR is a rapid pathway for resistance development, with the expansion of resistant populations of cleavers and the continuous generation of herbicide selection pressure, weeds evolve NTSR through complex mechanisms such as metabolic regulation and transmembrane transport, which is a combination of genetic and environmental selection.

The resistant populations AH-20, JS-15, and JS-22 displayed varied resistance toward pyroxsulam, tribenuron-methyl, bipyrazone, cypyrafluone, and isoproturon, whereas they exhibited sensitivity to the herbicides fluroxypyr, MCPA-sodium, carfentrazone-ethyl, and their mixtures. Although the AH-20, JS-15, and JS-22 populations possessed identical ALS resistance mutations, the levels of resistance to florasulam were different among them, as was their overall sensitivity to ALS-inhibiting herbicides. This may be caused by many factors, such as the frequency of the resistance mutations among populations, the possibility of other ALS resistance mutations (due to variations in the number of examined plants in this study), and the existence of NTSR (which was not specifically investigated in this study). It is noted that the SD-10 population (without known ALS resistance mutations) exhibited 10.74-fold resistance to florasulam compared to the susceptible population AH-9. However, the GR_{50} value for SD-10 was 2.04 g ai ha^{-1}, which is lower than the recommended rate for field use (3.0–4.5 g ai ha^{-1}) of florasulam in China. This may be caused by many factors such as the frequency of resistance mutations among populations, the possibility of other ALS resistance mutations, and the potential presence of NTSR. It is very likely that cleavers exhibit the basal metabolism of florasulam, and the key aspect lies in its different metabolic abilities. This is also supported by the cross-resistance identification of this SD-10 population, which exhibited resistance to the ALS inhibitor pyroxsulam and HPPD inhibitors bipyrazone and cypyrafluone, and resistance may be developing to isoproturon. Therefore, the herbicide resistance evolution by SD-10 should be closely followed and needs further investigation. On the whole, except for the ALS inhibitors, there is no pattern in the cross-resistance profiles of the four resistant populations tested here, which may also suggest that NTSR may be present and explain the resulting unpredictable resistance patterns that sometimes occur in cross-resistant populations (Délye 2013; Yu and Powles 2014a). This suggests that the genes responsible for NTSR might differ among populations, potentially leading to varying degrees of interactive resistance (Délye et al. 2010; Yu and Powles 2014b). NTSR is becoming a significant concern in various weed species, surpassing TSR in prevalence in certain cases, such as late watergrass [*Echinochloa phyllopogon* (Stapf) Koss.], rigid ryegrass (*Lolium rigidum*), false cleavers (*Galium spurium*), slender foxtail (*Alopecurus myosuroides* Huds.), and perennial ryegrass (*Lolium perenne*) (Wang et al. 2021). The NTSR mechanism of weeds refers to the reduction of phytotoxicity through physical and enzymatic mechanisms. The action of the effective lethal dose of a herbicide requires the herbicide to reach the target site in the plant body at a particular dose over a period of time that exerts lethality (Zhang et al. 2018). These mechanisms mainly include detoxification through metabolism, reduction of absorption into and transport within the plant, among which the detoxification metabolism of herbicides is considered the most common nontarget resistance mechanism (Bi and Dai 2020). Consequently, further investigation is necessary to elucidate the NTSR mechanisms in this species.

Practical Implications

In this research, we discovered that cleavers has evolved resistance to ALS inhibitors and the Trp-574-Leu mutation in the target *ALS* gene plays a role in the resistance evolution in most of the resistant populations, and the resistance driven by NTSR should be followed closely. This study also confirmed that some populations that are resistant to ALS inhibitors are also resistant to HPPD and PS II inhibitors, but other populations remain sensitive to MCPA-sodium, fluroxypyr, carfentrazone-ethyl, and their mixtures. Incorporating herbicides with different sites of action could be a more effective approach to combatting cleavers resistance to herbicides. Therefore, the promotion of integrated weed management program that includes various strategies is still recommended. At the same time, when controlling cleavers, relying solely on herbicides such as florasulam or MCPA-sodium may lead to increased herbicide resistance, thereby reducing their long-term effectiveness. Therefore, a diversified weed management strategy should be implemented. In addition to chemical control, nonchemical and agronomic measures can be integrated that optimize planting density by using crop residue mulching, and mechanical tillage. These approaches not only suppress the growth of cleavers but also improve field ecosystems and reduce the frequency of herbicide applications, thereby mitigating the risk of resistance. Such an integrated control strategy will promote sustainable weed management while maintaining agricultural productivity.

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Competing Interests. The authors declare they have no competing interests.

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