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Target-site mutation and enhanced metabolism endow resistance to nicosulfuron in a *Digitaria sanguinalis* population

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ABSTRACT

Digitaria sanguinalis is a competitive and annual grass weed that commonly infests crops across the world. In recent years, the control of *D. sanguinalis* by nicosulfuron has declined in Hebei Province, China. To determine the resistance mechanisms of *D. sanguinalis* to nicosulfuron, a population of *D. sanguinalis* where nicosulfuron had failed was collected from a maize field of Hebei Province, China. Whole-plant dose–response experiments demonstrated that the resistant population (HBMT-15) displayed 6.9-fold resistance to nicosulfuron compared with the susceptible population (HBMT-5). Addition of the glutathione S-transferase (GSTs) inhibitor 4-chloro-7-nitrobenzoxadiazole (NBD-Cl) significantly reduced the resistance level of the HBMT-15 population to nicosulfuron, and the GSTs activity of the HBMT-15 population was higher than the HBMT-5 population after nicosulfuron treatment. *In vitro* acetolactate synthase (ALS) enzyme experiments revealed that the nicosulfuron I_{50} value for the HBMT-15 population was 41 times higher than that of the HBMT-5 population. An Asp_{376} to Glu substitution in the *ALS* gene was identified in the HBMT-15 population. The HBMT-15 population had a moderate (2- to 4-fold) level of cross-resistance to three other ALS inhibitors (imazethapyr, pyroxsulam, and flucarbazone-sodium), but was susceptible to pyrithiobac-sodium. This study demonstrated that both an Asp_{376} to Glu substitution in the *ALS* gene and GSTs-involved metabolic resistance to ALS inhibitors coexisted in a *D. sanguinalis* population.

1. Introduction

Acetolactate synthase (ALS) is an essential enzyme in the biosynthesis pathway for leucine, isoleucine and valine (Duggleby and Pang, 2000). ALS is the target of five types of herbicides, including sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs), pyrimidinylthiobenzoates (PTBs), and sulfonylamino-carbonyl-triazolinones (SCTs) (Ray, 1984; Shaner et al., 1984; Gerwick et al., 1990; Chaleff and Mauvais, 1984). Due to the advantages of ALS inhibitors, such as low toxicity, high activity, and broad-spectrum weed control, these herbicides have been used continuously for more than thirty years. However, repeated and continuous use of ALS inhibitors causes high selection pressure on weeds; as a result, 171 weed species have evolved resistance to ALS inhibitors (Heap, 2023).

Target-site resistance (TSR) and non-target-site resistance (NTSR) are the main resistance mechanisms for ALS inhibitors (Délye, 2013). TSR is the predominant mechanism for ALS inhibitors resistance, and is mainly caused by amino acid substitutions in ALS, leading to insensitive to herbicide binding. Eight amino acid substitutions are reported in the ALS of weeds that can confer resistance to herbicides, including Ala_{122} (six mutations), Pro_{197} (ten mutations), Ala_{205}(two mutations), Asp_{376} (one mutation), Arg_{377} (one mutation), Trp_{574} (four mutations), Ser_{653} (three mutations), and Gly_{654} (two mutations) (Tranel et al., 2023).

Non-target site resistance (NTSR) mechanisms are involved in all other resistance mechanisms where no alteration to the target site is present (Délye, 2013). NTSR encompasses a range of diverse mechanisms such as enhanced metabolism, reduced penetration, and translocation. Among these, enhanced metabolism is known to endow resistance to ALS inhibitors in weeds (Yuan et al., 2007; Powles and Yu, 2010). Enhanced metabolism of herbicides in weeds is may involve cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), aldo keto reductases (AKR), oxidases, esterases, hydrolases, or peroxidases (Délye et al., 2013). P450s play an important role in conferring resistance to ALS inhibitors in *Sagittaria trifolia*, *Echinochloa*

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crus-gall, *Descurainia sophia*, *Alopecurus aequalis*, *Echinochloa phyllopogon*, *Ipomoea purpurea* and *Malachium aquaticum* (Feng et al., 2022; Shen et al., 2022; Zhao et al., 2022; Zhao et al., 2017a, 2017b; Yang et al., 2016; Iwakami et al., 2014). GSTs can also confer resistance to herbicides in weeds (Cummins et al., 2011), and studies have reported that *E. crus-gall*, *A. aequalis* and *M. aquaticum* have evolved resistance to ALS-inhibitors due to overexpression of GSTs genes (Feng et al., 2022; Zhao et al., 2017a, 2017b; Liu et al., 2018). Zou et al. (2022) find that increased GSTs activity was responsible for resistance of *S. trifolia* to bensulfuron-methyl (Zou et al., 2022).

More than one hundred species of *Digitaria* are recognized as weeds, among them, *Digitaria sanguinalis* has become one of the most difficult weeds to manage worldwide. *D. sanguinalis* has high fecundity, fast growth, high tillering ability and produces a large number of seeds. Strong survivability can allow it to infest all crops, non-crops, and even turf (Holm et al., 1977). A *D. sanguinalis* density of 2–128 plant m^{-2}, produces a yield loss in dry direct-seed rice is 6%–91% (Guo et al., 2023). Due to the overuse of herbicides, *D. sanguinalis* has evolved resistance to four modes of action of herbicides, including acetolactate synthase (ALS), acetyl-coenzyme A carboxylase (ACCase), enolpyruvyl shikimate phosphate synthase (EPSPS) and photosystem II (PSII). One mutation (Trp_{574} to Arg) in *ALS* and cytochrome P450s-involved metabolism afford resistance to ALS inhibitors; one mutation (Pro_{106} to His) in *EPSPS* endows glyphosate resistance; and *ACCase* gene overexpression confers resistance to ACCase inhibitors in *D. sanguinalis* (Yanniccari et al., 2022; Laforest et al., 2017; Mei et al., 2017; Li et al., 2017; Hidayat and Preston, 2001; Gdamski et al., 1996). To date, no other *ALS* mutations or glutathione *S*-transferases (GSTs) involved in metabolism have been found in *D. sanguinalis*.

Currently, *D. sanguinalis* is ranked the 2nd most noxious weed in 17 important weed species of farmland in China that cause reductions in crop production (Jin et al., 2021). Nicosulfuron has been used for the control of *D. sanguinalis* for more than thirty years in maize fields. In the past few years, growers have found that the control by nicosulfuron on *D. sanguinalis* was reduced in Hebei Province, China. This study aimed to (1) evaluate the resistance level of the *D. sanguinalis* population to nicosulfuron, (2) determine cross resistance to four ALS inhibitors, and (3) explore potential resistance mechanisms in the *D. sanguinalis* population.

2. Materials and methods

2.1. Plant materials

In Ningjin County, Hebei Province, China, in October 2019, seeds of the HBMT-15 population were collected from maize fields, where nicosulfuron control problems were observed. In September 2019, seeds of the HBMT-5 population, without exposure to herbicides, were collected from Fucheng County, Hebei Province, China (Table 1). After all seeds were air-dried naturally in the laboratory, they were placed in a low temperature and humidity storage cabinet. In June 2020, all seeds were dehulled into caryopses using emery paper for 30 s, and then planted in plastic tubs (9 × 9 cm), filled with a mixture of peat: sand (1:1). All plastic tubs containing seeds were transferred to a greenhouse (35 °C day (14 h) / 25 °C night (10*h*)), and when *D. sanguinalis* grew to the 2-leaf stage, they were thinned to five plants per tub.

Table 1
Digitaria sanguinalis collection locations and nicosulfuron application histories.

Collection locations	Populations	Latitude	Longitud	Estimated years of application
Fucheng	HBMT-5	N 37.91°	E 116.36°	Approximately 12 years
Ningjin	HBMT-15	N 37.68°	E 114.83°	Never

2.2. Whole-plant dose-response experiments with or without 4-chloro-7-nitrobenzoxadiazole (NBD-Cl)

Uniform seedlings of *D. sanguinalis* were treated with nicosulfuron (2-[(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl]-*N*,*N*-dimethylnicotinamide, 40 g L^{-1} OD, Ishihara Sangyo Kaisha Ltd., Japan), imazethapyr (5-ethyl-2-[(*RS*)-4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl]nicotinic acid, 5% AS, Shandong Cynda Chemical, China), flucarbazone-sodium (sodium 4,5-dihydro-3-methoxy-4-methyl-5-oxo-N-{[2-(trifluoromethoxy)phenyl]sulfonyl}-1H-1,2,4-triazole-1-carboximidate, 70% WG, Jiangsu Repont Agrochemical Ltd., China), pyrithiobac-sodium (sodium 2-chloro-6-(4,6-dimethoxypyrimidin-2-ylthio) benzoate, 80% TC, Kumiai Chemical Industry Ltd., Japan) and pyroxsulam (N-(5,7-dimethoxy[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)pyridine-3-sulfonamide, 4% OD, Corteva Agricultural Technology Ltd., USA) (Table 2) applied through a laboratory sprayer with a Teejet XR8003 flat fan nozzle delivering 450 L ha^{-1} at 0.3 MPa (3 WP-2000, Nanjing Research Institute for Agricultural Mechanization, Nanjing, National Ministry of Agriculture of China) at the 3-leaf stage. NBD-Cl was a commonly used inhibitor of GSTs that did not affect the normal growth of seedlings of *D. sanguinalis* (data not shown). Nicosulfuron (Table 2) was applied three days after application of 270 g a.i. ha^{-1} NBD-Cl (98%, Shanghai Aladdin Biochemical Technology Ltd., China) dissolved in a 0.1% aqueous solution of Tween-80 (98%, Beijing Solarbio Life Sciences Ltd., China). After herbicide treatments, all seedlings of *D. sanguinalis* were returned to the greenhouse. Every treatment contained four replications, and experiments were repeated twice. The fresh weight of aboveground shoots was recorded 21 days after herbicides treatments.

2.3. ALS gene sequencing

Plants of the HBMT-15 population that survived treatment with 60 g a.i. ha^{-1} nicosulfuron at the 3–4 leaf stage, and HBMT-5 without nicosulfuron treatment were used for DNA extraction. Total DNA was extracted from young leaves of the HBMT-15 and HBMT-5 populations using the Plant Genomic Kit (Beijing TIANGEN Biotech Ltd., China). Based on the *ALS* gene sequences of *D. sanguinalis* (GenBank: KX461957.1), two pairs of primers (Table 3) were designed to amplify the *ALS gene* of *D. sanguinalis* including eight reported resistance-endowing mutation sites (Li et al., 2017). The polymerase chain reaction (PCR) system included 1 μL template DNA of *D. sanguinalis*, 0.8 μL forward primer, 0.8 μL reverse primer, 10 μL 2 × Taq PCR Master Mix and 7.4 μL ddH_2O (APExBIO Technology Ltd., Houston, USA). The PCR system followed the procedure below: initial denaturation (94 °C) for 4 min; 32 cycles of denaturation (94 °C) for 30 s, annealing (55 °C) for 30 s, and extension (72 °C) for 60 s; and final extension (72 °C) for 5 min. The PCR product was purified with a Cowin Gel Extraction Kit (Cowin Biotech Ltd., Jiangsu, China) and sequenced by a technology company (Ruibo Biotech Ltd., Beijing, China). Sequence data were analyzed and assembled with DNAMAN 6.0 software (Lynnon Biosoft Ltd., Quebec, Canada).

2.4. In vitro ALS inhibition assay

When seedlings of *D. sanguinalis* were cultured to the 3-leaf stage as described in Sections 2.1. Approximately 5 g of young leaves of the HBMT-15 and HBMT-5 populations were separately harvested, immediately frozen in liquid nitrogen and then transferred to an ultralow temperature freezer (−80 °C). Extraction and purification of ALS, and *in vitro* herbicide inhibition assays were performed as described by Yu et al. (Yu et al., 2010). The frozen leaves were ground into powder in a mortar filled with liquid nitrogen and then homogenized in 10 mL of extraction buffer (Yu et al., 2010). The homogenate was filtered through four layers of Miracloth and centrifuged at 25000 ×*g* for 20 min at 4 °C. The supernatant was decanted into another centrifuge tube, brought to 50%

Table 2
List of herbicides used.

Herbicides	Chemical class[a]	Content&Formulations[b]	Manufacturer	Rate range of dose (g a.i. ha^{-1})
Nicosulfuron	SU	40 g L^{-1} OD	Ishihara Sangyo Kaisha, Japan	0, 30, 60, 120, 180, 240, 300, 360
Imazethapyr	IMI	5% AS	Shandong Cynda Chemical, China	0, 15, 30, 60, 120, 240
Pyroxsulam	TP	4% OD	Corteva Agricultural Technology, USA	0, 3.125, 6.25, 12.5, 25, 50
Pyrithiobac-sodium	PTB	80% TC	Kumiai Chemical Industry, Japan	0, 7.5, 15, 30, 60, 120
Flucarbazone-sodium	SCT	70% WG	Jiangsu Repont Agrochemical, China	0, 6.25, 12.5, 25, 50, 100

[a] Chemical class: SU: sulfonylureas, IMI: Imidazolinones, TP: triazolopyrimidines, PTB: pyrimidinylthiobenzoates, SCT: triazolinones.
[b] Formulations: OD: Oil Dispersion, AS: Aqueous Solutio, TC: Technical Material, WG: Water Dispersible Granule.

Table 3
Primers designed to amplify the ALS gene of *Digitaria sanguinalis*.

Primer	Sequence(5′-3′)	Targeted mutations[a]
1	F1 GGCGCGGACATCCTCGTCGA R1 TTTGGTCAGCTCATCCAGAACCTG	Ala122, Pro197, Ala205, Asp376, Arg377
2	F2 GTGGATAAGGCCGACCTGTTGCT R2 CTGCCATCACC(A/G) TCCAGGATCAT	Trp574, Ser653, Gly654

F: forward primer, R: reverse primer.
[a] Numbering of amino acids as per *Arabidopsis thaliana* ALS sequence.

ammonium sulfate saturation, placed in an ice–water system for 30 min, and then precipitated at 25000 ×*g* for 20 min at 4 °C. The supernatant was discarded, and the pellet was redissolved in a reaction buffer with a volume of 5 mL (Yu et al., 2010). The reaction system included enzyme extract (100 μL) and ALS-inhibitor solution (100 μL) and was treated at 37 °C for 60 min, and the reaction was stopped as described by Yu et al. (2010). ALS activity was measured colorimetrically (530 nm) with a UV/Vis spectrophotometer (Molecular Devices Ltd., USA) by quantifying acetoin production, and the protein concentration of extracts was determined using the method of Bradford (Bradford, 1976). Nicosulfuron concentrations (technical-grade, 95% purity, Jiangsu Repont Agrochemical Ltd., China) used for the ALS activity assay were 0, 0.001, 0.01, 0.1, 1, 10 and 100 μM for the HBMT-5 population, and 0, 0.01, 0.1, 1, 10, 100 and 1000 μM for the HBMT-15 population. Every treatment contained three replications, and the experiments were repeated two times.

2.5. GSTs activity assay

At the 3-leaf stage, the HBMT-15 and HBMT-5 populations were treated with 60 g a.i. ha^{-1} of nicosulfuron. Fresh leaves were separately collected from the HBMT-15 and HBMT-5 populations at 0, 24, 48, 72, 96 and 120 h after nicosulfuron treatment, immediately frozen in liquid nitrogen and then transferred to an ultralow temperature freezer (−80 °C). GSTs extraction and activity assays of *D. sanguinalis* were performed according to the GSTs Activity Assay Kit (Beijing Solarbio Life Sciences Ltd., China). Approximately 0.1 g frozen leaves were ground into powder in a mortar filled with liquid nitrogen and homogenized in 1 mL of buffer I (from the kit) on ice. The mixture solution was centrifugated at 8000 ×*g* for 10 min at 4 °C, and the supernatant was decanted into another centrifuge tube following by diluting 50 times with ddH_2O on ice. The reaction system containing the diluted supernatant (20 μL), buffer II (180 μL) and buffer III (20 μL) was heated at 37 °C for 5 min. The absorbance of the treatment was measured (340 nm) with a microplate reader (Bio-Tek Instrument Ltd., Vermont, USA). Every treatment had three replications, and experiments were repeated twice.

2.6. Statistical analyses

There were no significant (*t*-test, $p > 0.05$) differences between the repeated experiments of whole-plant dose-response experiments, *in vitro* ALS inhibition assays and GSTs activity assays, so the data were pooled. For whole-plant dose-response experiments and *in vitro* ALS activity assays, data were expressed as percentages of untreated control and fitted to the four-parameter log-logistic equation described below (Seefeldt et al., 1995).

$$y = c + (d - c)/[1 + exp.[b(log(x) - log(e)]$$

In the equation, *y* represents the response at herbicide concentrations x, *c* and *d* are asymptotic values at the lower and upper limits, respectively, *b* is the slope, and *e* is the effective dose causing a 50% reduction in growth (GR_{50}) or 50% inhibition of *in vitro* ALS activity (I_{50}). Regression analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA). Resistance indexes (RI) from whole-plant dose-response and *in vitro* ALS assays were calculated as resistant to susceptible population ratios of GR_{50} or I_{50}.

3. Results

3.1. Whole-plant dose-response experiments with or without NBD-Cl

The *D. sanguinalis* populations differed in the fresh weights of shoots in response to the herbicides used (Table 2). At the field-recommended dose of nicosulfuron (60 g a.i. ha^{-1}), the fresh weights of shoots of the HBMT-5 population was reduced by 91% compared to the untreated, whereas the HBMT-15 population had no reduction in fresh weight (Fig. 1A and Table 4). The GR_{50} values of the HBMT-5 population and HBMT-15 population were 31.8 and 218.5 g a.i. ha^{-1}, respectively, and the resistance index (RI) was 6.9, indicating that the HBMT-15 population had evolved resistance to nicosulfuron. The HBMT-15 population had also evolved resistance to imazethapyr, flucarbazone-sodium and pyroxsulam (RI values ranged from 2 to 4) (Fig. 1B, C, D and Table 4), but this population was still susceptible to pyrithiobac-sodium (Fig. 1E and Table 4).

The GSTs inhibitor NBD-Cl applied alone at a dose of 270 g a.i. ha^{-1} did not affect growth of the HBMT-5 and HBMT-15 populations (data not shown). For the HBMT-5 population, the GR_{50} values for nicosulfuron were 29.7 and 31.8 g a.i. ha^{-1} with or without NBD-Cl, respectively. In contrast, the GR_{50} values of the HBMT-15 population with NBD-Cl were reduced by approximately 51% compared with those without NBD-Cl (Table 4), indicating that GSTs could contribute to resistance to nicosulfuron in HBMT-15.

3.2. ALS gene sequencing

Fragment sequences of 946 and 733 bp were amplified by primer pair 1 and primer pair 2, respectively, from 20 individuals of the HBMT-5 population and HBMT-15 population (surviving nicosulfuron treatment). One 1679 bp DNA sequence of the *ALS* gene was assembled from the above fragment sequences, containing five highly conserved regions from A to E, in which eight known mutation sites could confer resistance to ALS inhibitors. The results of sequence comparison indicated that the *ALS* genes of the HBMT-5 and HBMT-15 populations were approximately 99.5% and 99.7% similar to that of *D. sanguinalis,* respectively. (GenBank accession KX461957.1). In the HBMT-15 population, an aspartate was changed to glutamate at position 376 (Asp_{376} to Glu) of

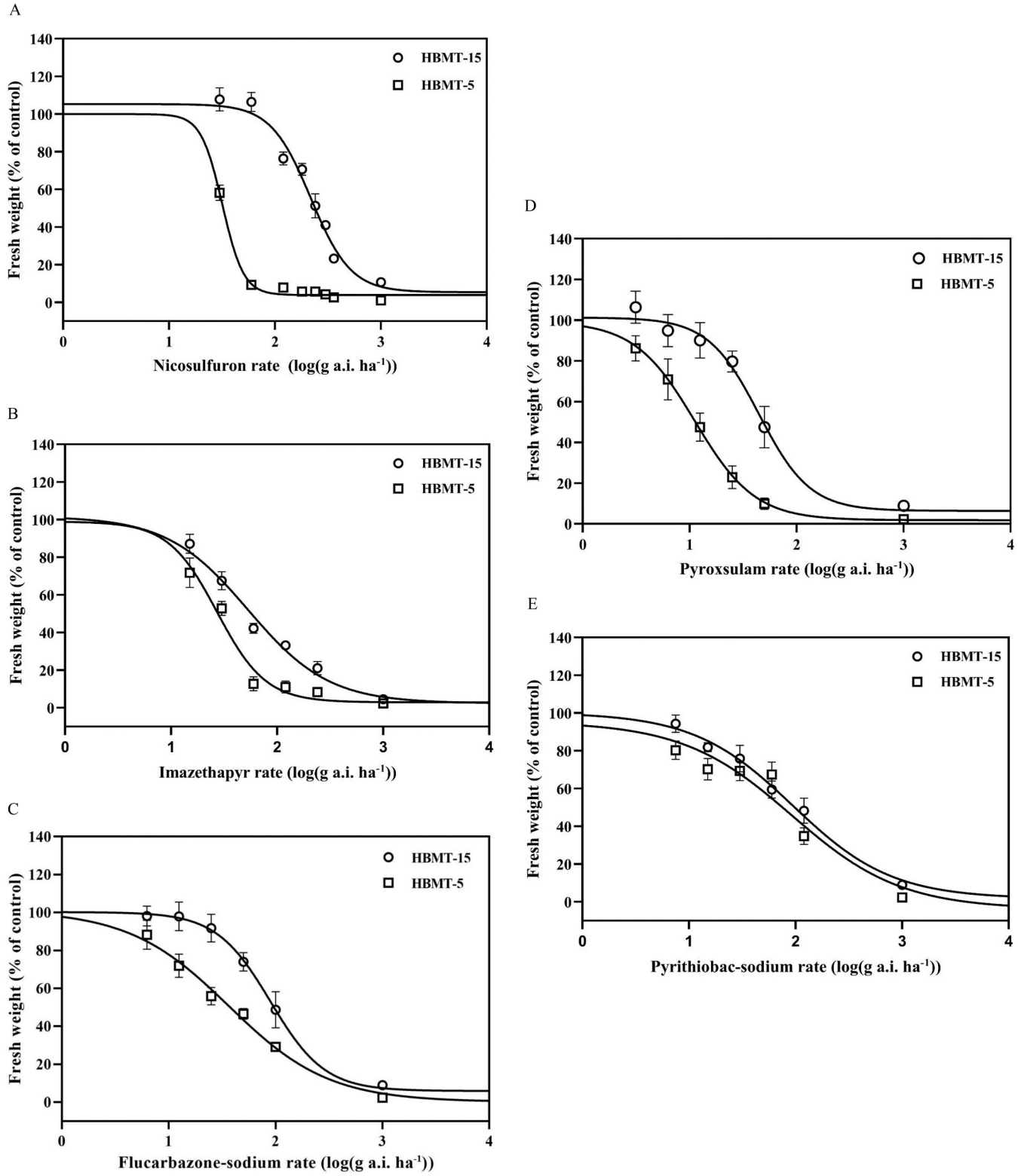

Fig. 1. Dose–response curve for above ground fresh weights of the susceptible population HBMT-5 (□) and resistant population HBMT-15 (○) of *Digitaria sanguinalis* treated with a range of (A) nicosulfuron, (B) imazethapyr, (C) flucarbazone-sodium, (D) pyroxsulam and (E) pyrithiobac-sodium doses. Each data point represents the mean fresh weight (percentage of control) ± SE of two repeated experiments.

ALS resulting from a point mutation (GAT to GAA). However, other seven positions (Ala_{122}, Pro_{197}, Ala_{205}, Arg_{377}, Trp_{574}, Ser_{653}, and Gly_{654}) were not found to have any mutations in the HBMT-15 population. This amino acid substitution at position 376 of *ALS* is contributing to nicosulfuron resistance in *D. sanguinalis*.

3.3. In vitro ALS inhibition assay

In the absence of nicosulfuron, the total ALS activity was 4.9 and 5.3 nmol acetoin mg^{-1} protein min^{-1} in the HBMT-5 and HBMT-15 populations, respectively, and there was no significant difference between

Table 4
GR_{50} values of *Digitaria sanguinalis* populations treated with herbicides and ratios (R/S) of the GR_{50} values of resistant to susceptible plants.

Herbicides	GR_{50} (g a.i. ha^{-1}) [a]		R/S ratio of GR_{50}
	HBMT-15	HBMT-5	
Nicosulfuron	218.5 ± 39.7	31.8 ± 2.8	6.9
Nicosulfuron+NBD-Cl	105.5 ± 25.7	29.7 ± 2.4	3.6
Imazethapyr	53.2 ± 10.7	27.1 ± 5.9	2.0
Pyroxsulam	44.5 ± 11.9	11.2 ± 3.2	4.0
Pyrithiobac-sodium	98.2 ± 40.8	92.3 ± 46.9	1.1
Flucarbazone-sodium	89.7 ± 29.1	36.6 ± 12.6	2.5

[a] GR_{50}: herbicide dose causing a 50% growth reduction of population. Each value represents the mean ± standard error.

the HBMT-15 and HBMT-5 populations (Table 5). Significant differences in the dose-response of ALS activity were found in the HBMT-15 and HBMT-5 populations after adding nicosulfuron (Fig. 2). The HBMT-5 population had an I_{50} value of 0.58 μM nicosulfuron; however, the I_{50} value for the HBMT-15 population was 23.8 μM (R/S = 41) (Table 5).

3.4. GSTs activity assay

No differences in GSTs activity were noted between the HBMT-5 and HBMT-15 populations before nicosulfuron treatment. After nicosulfuron treatment, GSTs activity of the HBMT-15 population rapidly increased during 0–48 HAT and gradually decreased during 48–120 HAT. However, GSTs activity of the HBMT-5 population displayed a continuous downward trend during 0–120 HAT. The HBMT-15 population showed 1.3- to 1.7-fold higher GSTs activity compared with the HBMT-5 population during 24–120 HAT (Fig. 3).

4. Discussion

This study investigated the resistance level and mechanism of suspected resistant *D. sanguinalis* to nicosulfuron, which demonstrated that *D. sanguinalis* evolved resistance conferred by two coexisting resistance mechanisms to nicosulfuron in maize fields of Hebei Province, China. The results show a commercial rate of 40–60 g a.i. ha^{-1} normally used to control *D. sanguinalis* could not control the HBMT-15 population (Fig. 1 and Table 4), which is consistent with the description from local growers. Globally, resistance of *D. sanguinalis* has become a serious problem. In maize fields in China, Mei et al. (2017) suggested almost twenty-six *D. sanguinalis* populations have evolved resistance to nicosulfuron, and Li et al. (2017) showed that *D. sanguinalis* displayed broad resistance to three ALS inhibitors. Furthermore, in onion fields of Australia and Canada, *D. sanguinalis* evolved cross-resistance to ACCase inhibitors (Hidayat and Preston., 1997; Laforest et al., 2017) and multiple resistance to imazethapyr (Hidayat and Preston., 2001). More seriously, *D. sanguinalis* was even identified as resistant to glyphosate in soybean fields of Argentina (Yanniccari et al., 2022).

Table 5
Total ALS activity in absence of herbicides, and I_{50} values determined partially purified ALS enzyme isolated from the susceptible population HBMT-5 and resistant populations HBMT-15 of *Digitaria sanguinalis* in presence of nicosulfuron.

Populations	Total ALS activity (nmol acetoin mg^{-1} protein min^{-1})	R/S	I_{50} [a] (μM)	R/S
HBMT-5	4.9 ± 0.4 a[c]	–	0.58 ± 0.08	–
HBMT-15	5.3 ± 0.2 a	1.1	23.8 ± 7.4	41.0

[a] I_{50}: herbicide dose causing 50% inhibition of ALS activity. Each value represents the mean ± standard error.
[c] Means with different letters in the same column for three populations are significantly ($P = 0.05$).

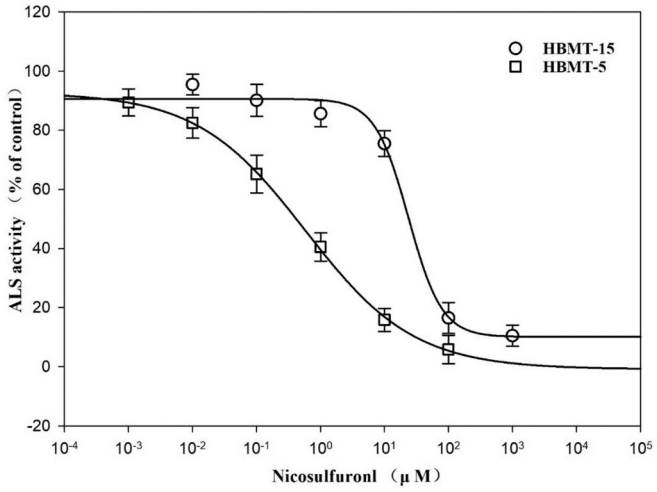

Fig. 2. *In vitro* ALS enzyme activity of nicosulfuron. Each data point represents the mean activity (percentage of control) ± SE of two repeated experiments.

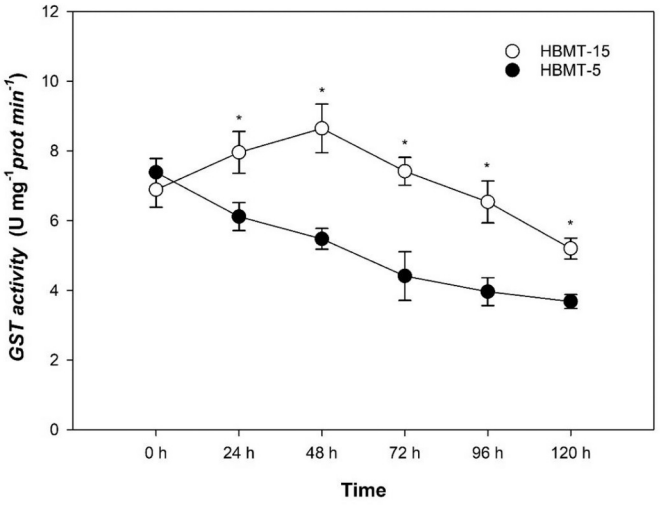

Fig. 3. Changes in GSTs activity in HBMT-5 and HBMT-15 population after nicosulfuron treatment. Each data point represents the mean activity ± SE of two repeated experiments. The values labelled with asterisks above the HBMT-15 curve indicate significant difference from HBMT-5 ($P < 0.05$).

Notably, resistance to imazethapyr, flucarbazone-sodium and pyroxsulam appeared without prior selection with these herbicides. In addition, imazethapyr is registered for annual weed control in soybean fields; however, flucarbazone-sodium and pyroxsulam are not registered for *D. sanguinalis* control. Resistance to the above three ALS inhibitors suggests that the main reason for cross-resistance evolution in *D. sanguinalis* was selection pressure by frequent application of nicosulfuron. Examples of cross-resistance to ALS inhibitors by *ALS* gene mutation and metabolic enzyme metabolism have been previously reported in *Setaria viridis* and *Amaranthus retroflexus* (Huang et al., 2021; Huang et al., 2020). In *E. crus-galli* populations, *ALS gene* mutation and GSTs-involved metabolism evolved resistance to ALS inhibitors (Feng et al., 2022), which is similar to our findings.

Sequencing of the *ALS gene* from the HBMT-15 population demonstrated a mutation that changed Asp_{376} to Glu, indicating that it played a major role in nicosulfuron resistance. The results of *in vitro* ALS experiments indicated that the ALS of the HBMT-15 population was more insensitive to nicosulfuron than that of the HBMT-5 population (Fig. 2 and Table 5). Asp_{376} to Glu substitution has been demonstrated to

endow resistance in *S. viridis*, *Galium aparine*, *Lolium perenne* and *A. retroflexus* (Huang et al., 2021; Deng et al., 2019; Menegat et al., 2016; Huang et al., 2016). In addition, other studies have also shown that Asp_{376} substitutions can lead weeds to evolve broad resistance to SUs, TPs, SCTs and IMIs (Tranel et al., 2023). Asp_{376} to Glu substitution identification in the HBMT-15 population was indicated to contribute to cross-resistance to nicosulfuron (SUs), pyroxsulam (TPs), flucarbazone-sodium (SCTs) and imazethapyr (IMIs) (Fig. 1 and Table 4), which was also consistent with previous reports (Ashigh et al., 2009; Deng et al., 2019).

NTSR can result in weeds evolving resistance to herbicides with different mechanisms of action (Feng et al., 2022). NBD-Cl, a commonly used GSTs inhibitor, was used to examine GSTs-mediated metabolic resistance to mefenacet, penoxsulam, trifluralin, quizalofop-p-ethyl and fomesafen, and the results indicated that NBD-Cl could reverse resistance to these herbicides (Cai et al., 2022; Feng et al., 2022; Varanasi et al., 2018; González-Torralva and Norsworthy, 2021). In the present study, NBD-Cl reduced resistance of the HBMT-15 population to nicosulfuron (Table 4), indicating that GSTs played an important role in resistance to nicosulfuron in the HBMT-15 population.

The GSTs activity of the resistant *E. crus-galli* population was higher than that of the susceptible population after the application of mefenacet, suggesting that induction of GSTs may contribute to mefenacet resistance in the resistant *E. crus-galli* population (Cai et al., 2022), which is similar to the GSTs activity assay in the present study (Fig. 3). The HBMT-15 and HBMT-5 populations had comparable GSTs activities in the absence of nicosulfuron application, therefore overexpression of GSTs in the HBMT-15 population is not likely. However, the GSTs activity of the HBMT-15 population was higher than that of the HBMT-5 population after nicosulfuron treatment, which may be related to the inducible expression of one or more GSTs by the herbicide. Further study should be conducted to identify and validate the genes involved in metabolic resistance in *D. sanguinalis*.

In conclusion, this study documented a case of both target-site based (Asp_{376} to Glu substitution) and GSTs-involved metabolism resistance to ALS inhibitors coexisting in a *D. sanguinalis* population collected from maize fields of Hebei Province in China. The evolution of target-site resistance and GSTs-involved metabolism resistance in *D. sanguinalis* presents a critical challenge to effective weed management. Thus, it is important to prevent and control *D. sanguinalis* before it becomes more threatening to crops.

Author contributions

Guiqi Wang and Xiaomin Liu conceived the idea and designed the experiments. Bochui Zhao, Xian Xu, Binghua Li and Zhizun Qi participated in various parts of the experiments. Jinan Huang and Ali Hu helped in collecting data. Bochui Zhao wrote the manuscript. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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