



## Diverse ALS mutations and cross-and multiple-resistance to ALS and EPSPS inhibitors in flucarbazone-sodium-resistant *Bromus japonicus* populations from Hebei province, China

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### ABSTRACT

Japanese brome (*Bromus japonicus*) has become one of the main weeds in wheat fields in Hebei province of China and causes a large decrease of wheat production. A total of 44 putative resistant and 2 susceptible Japanese brome populations were collected in the 2021/2022 crop season from Hebei province of China to determine resistance levels to flucarbazone-sodium and to investigate the diversity of acetolactate synthase (ALS) mutations, as well as to confirm the cross-and multiple-resistance levels to ALS and EPSPS (5-enolpyruvate shikimate-3-phosphate synthetase) inhibitors. Whole plant bioassay results showed that 15 out of 44 populations tested or 34% were resistant to flucarbazone-sodium. The resistance indices of Japanese brome to flucarbazone-sodium ranged from 43 to 1977. The resistant populations were mainly distributed in Baoding and Shijiazhuang districts, and there was only one resistant population in Langfang district. Resistant Japanese brome had diverse ALS mutations, including Pro-197-Ser, -Thr, -Arg and Asp-376-Glu. The incidence of Pro-197-Ser mutation was the highest at 68%. Application of the CYP450 inhibitor malathion suggested that CYP450 was involved in metabolic resistance in a population without an ALS mutation. The population with Pro-197-Thr mutation evolved weak cross-resistance to mesosulfuron-methyl and pyroxsulam, and it is in the process of evolving multiple-resistance to glyphosate.

### 1. Introduction

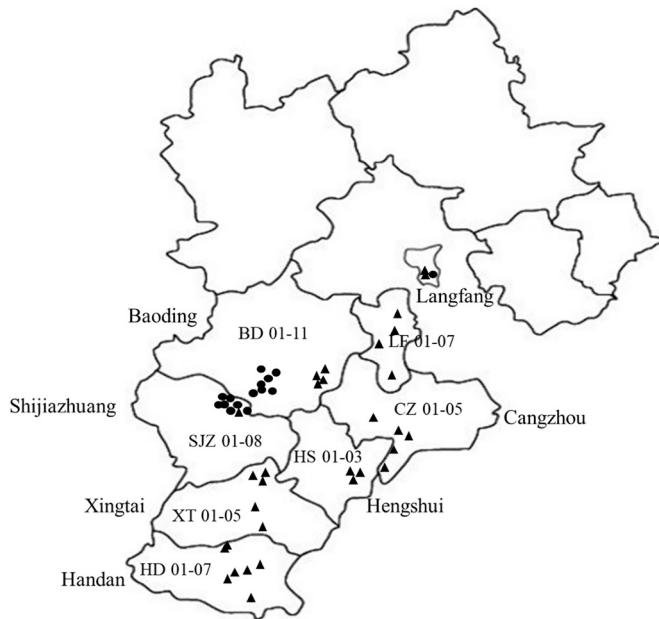
Japanese brome (*Bromus japonicus*), a self-pollinated winter annual grass, belonging to Gramineae family, is widely distributed in wheat fields and wastelands in northern China (Lan et al., 2022). Japanese brome has become one of the main weeds in wheat fields where it competes with the crop (Li et al., 2016). Seeds of Japanese brome usually germinate in September and October and flowering occurs in early May (Baskin and Baskin, 1981). Each plant can produce about 2000 seeds, which can be easily spread by wind or water because of their light weight (Wang, 1986). Japanese brome at a density of 24 plants m<sup>-2</sup> to 532 plants m<sup>-2</sup>, resulted in 15% to 30% reduction of wheat yields (Sarani et al., 2021). At present, Japanese brome has become a serious threat to wheat production and farmers' income (Johnson et al., 2018; Metier et al., 2020).

Flucarbazone-sodium is a useful herbicide to control Japanese brome and belongs to acetolactate synthase (ALS) inhibiting herbicides (Saleh, 2022). It was registered in China in 2008 and has been applied in wheat fields to control Japanese brome for about 15 years. However, due to excessive dependence on flucarbazone-sodium, Japanese brome has developed resistance to this herbicide (Lan et al., 2022; Li et al., 2022b). According to reports, as early as the beginning of this century, Chinese and German weed scientists had found resistant Japanese brome species in wheat fields in the North China plain (Menegat et al., 2011).

Elucidating the resistant mechanisms of weeds to herbicides is very important for developing weeds control technologies. At present, resistant mechanisms to ALS-inhibiting herbicides are divided into two kinds: target-site resistance (TSR) and nontarget-site resistance (NTSR) (Yu and Powles, 2014; Ntoanidou et al., 2019; Zhu et al., 2023). TSR mechanisms occur due to mutations in the conservative region of ALS.

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**Fig. 1.** The map of collection sites of Japanese brome (*Bromus japonicus*) populations. Collection sites of the 46 Japanese brome populations (LF 01–07, BD 01–11, CZ 01–05, SJZ 01–08, HS 01–03, XT 01–05, HD 01–07) studied in this work. The map is Hebei Province of China. The triangle represented susceptible Japanese brome populations, and the dot represented resistant Japanese brome populations.

ALS mutations lead to the obstruction of herbicides entry into the active center of ALS enzyme, or the reduction of herbicides binding ability with the target enzyme (Zhang et al., 2017; Zhang et al., 2021; Zou et al., 2022). So far, the mutation sites which confer resistance to ALS-inhibiting herbicides in weeds are Ala122, Pro197, Ala205, Phe206, Asp376, Arg377, Trp574, Ser653 and Gly654 (Yu and Powles, 2014; Xu et al., 2015; Li et al., 2022a; Milani et al., 2023), and 27 amino acid mutations of ALS have been reported in 172 weed species (Heap, 2023). By contrast, NTSR mechanisms confer resistance to ALS-inhibiting herbicides through cytochrome P450 monooxygenases (CYP450) genes, glutathione S-transferases (GSTs) genes and ABC transporter genes (ABC), which increase metabolism of herbicides (Yang et al., 2016; Torra et al., 2021; Zhang et al., 2021; Bai et al., 2022). Among of them, many CYP450 genes are identified in several herbicide-resistant weed species (Dimaano and Iwakami, 2021), for example *Descurainia sophia* (Yang et al., 2016), *Capsella bursa-pastoris* (Zhang et al., 2021), *Myosoton aquaticum* (Bai et al., 2019), *Lolium rigidum* (Torra et al., 2021) and *Echinochloa crus-galli* (Pan et al., 2022).

Japanese brome resistant mechanisms to flucarbazone-sodium have been studied only in two populations from Hebei Province and Tianjin of China, which have Pro-197-Ser and Asp-376-Glu mutations respectively (Lan et al., 2022; Li et al., 2022b). However, we found many other Japanese brome-resistant populations in wheat fields, and the extent of resistance and the resistance mechanisms of Japanese brome different populations should be studied. In this paper, we: (1) tested the resistance levels of various Japanese brome populations to flucarbazone-sodium and clarified the distribution of resistance in Hebei Province of China; (2) identified the mutations in ALS of resistant populations; (3) investigated the cross - and multiple-resistance of Japanese brome with Pro-197-Thr mutation to ALS and EPSPS inhibitors.

## 2. Materials and methods

### 2.1. Plant materials

Mature seeds of 44 suspected resistant Japanese brome populations

were harvested randomly from winter wheat fields of the mid- and southern parts of Hebei province in China (Fig. 1). Seeds of HD 01 and HD 02 populations were collected from uncultivated lands of Handan in Hebei province where flucarbazone-sodium was never applied and were used as susceptible controls in this study (Fig. 1). The seeds were collected in the 2021/2022 crop season. Seeds of each Japanese brome population were dried and stored in paper bag at room temperature until dormancy release. Seeds were sown in  $11 \times 11 \times 10$  cm plastic pots filled with a mixture of peat: sand (1:1), kept in a controlled climate chamber at  $25^{\circ}\text{C}/22^{\circ}\text{C}$  (day/night) with 16 h photoperiod with light intensity of 20,000 Lux. All the pots were watered once a week. Each pot was thinned to 5 seedlings before herbicides were applied.

### 2.2. Resistance testing

Flucarbazone-sodium (4,5-dihydro-3-methoxy-4-methyl-5-oxo-N-((2-(trifluoromethoxy)phenyl)sulfonyl)-1H-1,2,4-triazole-1-carboxamide sodium salt, 10%OD, Dengfeng Jinbo Pesticide Chemical Ltd., China) was applied at the 3 - to 4 - leaf stage of Japanese brome plants using a cabinet sprayer equipped with a Teejet XR8003 flat fan nozzle calibrated to spray  $400 \text{ L ha}^{-1}$  at 275 kPa. The doses of flucarbazone-sodium used were 0, 2.1, 21, 210, 2100, 6300 g a.i.  $\text{ha}^{-1}$ . Seedlings were returned to the chamber and harvested 28 days after treatment. Shoot fresh weight was measured. Each treatment had three replicates and the experiment was repeated twice.

### 2.3. ALS gene sequencing

According to the partial ALS gene sequence of *Bromus tectorum* (AF488771), one pair of primers (forward: 5' - TCCGAGCCCCG-CAAGGGCGC - 3'; reverse: 5' - CCTGCCATCACCTTCCATGA - 3') were designed to amplify 1720 bp partial coding region of ALS gene of Japanese brome populations, which contained Ala122, Pro197, Ala205, Phe206, Asp376, Arg377, Trp574, Ser653 and Gly654 mutation sites previously reported in ALS-resistant weeds.

Genomic DNA was extracted from 0.1 g of fresh leaf material at the 4-leaf stage from three individual plants of each population. The DNA was isolated according to the instructions of the DNA extraction kit (Tian Gen Biotechnology Company Ltd.). The PCR mixture contained 1  $\mu\text{L}$  DNA template (100 ng), 0.5  $\mu\text{L}$  of each primer (10 pmol  $\mu\text{L}^{-1}$ ), 12.5  $\mu\text{L}$  2Pfxmix (Jiangsu Kangwei Century Biotechnology Company Ltd.), and 10.5  $\mu\text{L}$  ddH<sub>2</sub>O to reach the final 25  $\mu\text{L}$  volume. The PCR program was denaturation at  $95^{\circ}\text{C}$  for 3 min; 33 cycles of 1 min at  $95^{\circ}\text{C}$ , 30 s at  $58^{\circ}\text{C}$ , and 1 min at  $72^{\circ}\text{C}$ ; 10 min at  $72^{\circ}\text{C}$  and finally hold at  $4^{\circ}\text{C}$ .

The ALS PCR products were isolated from agarose gel using Easy Pure Quick Gel Extraction Kit (Jiangsu Kangwei Century Biotechnology Company Ltd.). Then the PCR products were linked to the EASY-T1 cloning vector and transformed into Trans1-T1 Phage Resistant Chemically Competent Cell for cloning (TransGen Biotechnology Company Ltd.). The transgenic plasmids were sequenced by the Beijing Genomics Institute using an ABI Prism 3730XL DNA sequencer. The mutations in the conserved domain of partial ALS sequence of Japanese brome were analyzed using the DNAMAN software package (Version 6.0.3.48, Lynnon Biosoft, Canada) by alignment with the ALS sequence of *Arabidopsis thaliana* (NP\_190425).

### 2.4. Metabolic resistance bioassay

Metabolic resistance of SJZ 05 population without ALS mutations to flucarbazone-sodium was investigated by using the cytochrome P450 inhibitor malathion (o,o-dimethyl s-(1,2-dicarbethoxyethyl) dithiophosphate, 45%EC, Shandong Caoda Chemical Ltd., China). HD 02 population was used as susceptible control. Preliminary experiments showed that malathion applied at  $2000 \text{ g a.i. ha}^{-1}$  did not inhibit the growth of seedlings. Malathion was applied 1 h before flucarbazone-sodium treatment using the sprayer described above. The experiment

**Table 1**

Herbicides and their doses sprayed in the cross - and multiple - resistance bioassays.

Groups	Herbicides	Doses (g a.i. $\text{ha}^{-1}$ )
ALS inhibitor	Mesosulfuron-methyl 30 g $\text{L}^{-1}$ OD	0, 1.5, 15, 150, 1500, 3000
	Pyroxasulam 7.5% OD	0, 1.41, 14.1, 141, 1410, 2820
EPSPS inhibitor	Glyphosate-isopropylammonium	0, 150, 1500, 6000, 12,000, 24,000
	41% AS	

ALS, acetolactate synthase; EPSPS, 5-enolpyruvate shikimate-3-phosphate synthetase. OD, oil dispersion; AS, aqueous solution.

method was the same as section 2.2.

### 2.5. Cross - and multiple-resistance bioassay

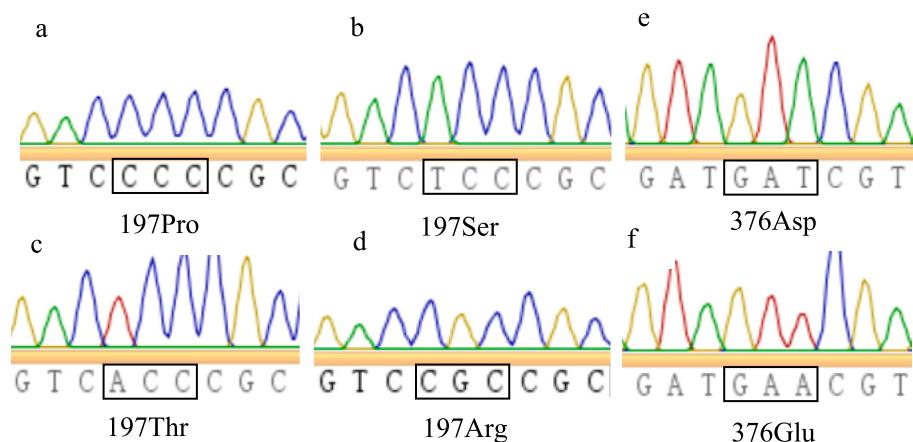
The highest resistant population SJZ 07 with Pro-197-Thr mutation was choosed to do the cross - and multiple-resistance bioassay. HD 02 population was used as susceptible control. Mesosulfuron-methyl (methyl 2-[3-(4,6-dimethoxyypyrimidin-2-yl)ureidosulfonyl]-4-methanesulfonamidoethylbenzoate, 30 g  $\text{L}^{-1}$  OD, Guangdong Haode Crop Technology Ltd., China) and pyroxasulam (N-(5,7-dimethoxy[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)-3-pyridinesulfonamide, 7.5% OD, Kedihua Agricultural Technology Ltd., China) were used to evaluate the cross-resistance, and glyphosate-isopropylamine (*N*-phosphomethylglycine, 41% AS, Qiaochang Modern Agriculture Ltd., China) was used to evaluate the multiple-resistance. The doses of herbicides were as shown in Table 1. The

**Table 2**

The resistance levels of Japanese brome (*Bromus japonicus*) populations to flucarbazone-sodium and diversified ALS mutations.

Populations	Biotypes	GR <sub>50</sub> (g a.i. $\text{ha}^{-1}$ )	R/S	Amino acid mutations of ALS		
				First Plant	Second Plant	Third Plant
BD 01	R	3874.0 ± 89.0	1845	Pro197Ser	Pro197Thr	Pro197Ser
BD 02	R	1585.9 ± 24.3	755	Pro197Ser	Pro197Ser	Pro197Ser
BD 03	R	3614.9 ± 55.3	1721	Pro197Ser	Pro197Ser	Pro197Ser
BD 04	R	2436.8 ± 19.8	1160	Pro197Ser	Pro197Ser	Pro197Ser
BD 05	R + S	886.9 ± 4.1	422	N	N	Pro197Arg
BD 06	R + S	836.2 ± 11.8	398	N	N	Pro197Arg
BD 07	R	1618.0 ± 92.3	770	Pro197Thr	Pro197Thr	Asp376Glu
BD 08	S	<2.1	1	N	N	N
BD 09	S	<2.1	1	N	N	N
BD 10	S	<2.1	1	N	N	N
BD 11	S	<2.1	1	N	N	N
SJZ 01	R	3714.2 ± 29.2	1769	Pro197Ser	Pro197Ser	Pro197Ser
SJZ 02	R	1443.7 ± 46.8	687	Pro197Ser	Pro197Thr	Pro197Ser
SJZ 03	R	2859.8 ± 38.1	1362	Pro197Ser	Pro197Ser	Pro197Ser
SJZ 04	R	2004.2 ± 84.8	954	Pro197Ser	Pro197Ser	Pro197Ser
SJZ 05	R	90.8 ± 3.3	43	N	N	N
SJZ 05 + M	R	30.3 ± 0.6	14	N	N	N
SJZ 06	R	1652.1 ± 61.9	787	Pro197Ser	Pro197Ser	Pro197Ser
SJZ 07	R	4151.4 ± 204.6	1977	Pro197Thr	Pro197Thr	Pro197Thr
SJZ 08	S	<2.1	1	N	N	N
XT 01	S	<2.1	1	N	N	N
XT 02	S	<2.1	1	N	N	N
XT 03	S	<2.1	1	N	N	N
XT 04	S	<2.1	1	N	N	N
XT 05	S	<2.1	1	N	N	N
LF 01	S	<2.1	1	N	N	N
LF 02	R	131.4 ± 11.3	63	Pro197Ser	Pro197Ser	Pro197Ser
LF 03	S	<2.1	1	N	N	N
LF 04	S	<2.1	1	N	N	N
LF 05	S	<2.1	1	N	N	N
LF 06	S	<2.1	1	N	N	N
LF 07	S	<2.1	1	N	N	N
HS 01	S	<2.1	1	N	N	N
HS 02	S	<2.1	1	N	N	N
HS 03	S	<2.1	1	N	N	N
CZ 01	S	<2.1	1	N	N	N
CZ 02	S	<2.1	1	N	N	N
CZ 03	S	<2.1	1	N	N	N
CZ 04	S	<2.1	1	N	N	N
CZ 05	S	<2.1	1	N	N	N
HD 01	S	<2.1	1	N	N	N
HD 02	S	<2.1	1	N	N	N
HD 02 + M	S	<2.1	1	N	N	N
HD 03	S	<2.1	1	N	N	N
HD 04	S	<2.1	1	N	N	N
HD 05	S	<2.1	1	N	N	N
HD 06	S	<2.1	1	N	N	N
HD 07	S	<2.1	1	N	N	N

R was resistant biotype and S was susceptible biotype. R + S was a mixed biotype with resistant and susceptible plants. M was cytochrome P450 inhibitor malathion at dose of 2000 g a.i.  $\text{ha}^{-1}$ . GR<sub>50</sub> was the herbicide dose that induced 50% fresh weight reduction of Japanese brome populations. The R/S index was GR<sub>50</sub>(R)/GR<sub>50</sub>(S). At the dose of 2.1 g a.i.  $\text{ha}^{-1}$  of flucarbazone-sodium, the fresh weight inhibition of susceptible Japanese brome was over 50%, so 2.1 g a.i.  $\text{ha}^{-1}$  was selected as the GR<sub>50</sub> of susceptible populations (Cui et al., 2011). Each GR<sub>50</sub> value represented the mean ± standard error.



**Fig. 2.** Diversified ALS mutations in resistant Japanese brome (*Bromus japonicus*) populations. (a) and (e) were the chromatogram of the nucleotide sequences of ALS in susceptible Japanese brome populations, the others were the chromatogram of the nucleotide sequences of ALS in resistant Japanese brome populations.

experiment method was the same as above described in section 2.2.

#### 2.6. Statistical analysis

The GR<sub>50</sub> was calculated using the following equation:

$$y = C + \frac{D - C}{1 + \exp\{b[\log(x) - \log(\text{GR}_{50})]\}}$$

where y was the seedling fresh weight at flucarbazone-sodium dose x, b was the slope at the GR<sub>50</sub>, C and D were the minimum and maximum plant fresh weight as a percentage of the untreated control. GR<sub>50</sub> was the herbicide dose causing 50% inhibition in above ground fresh weight. Statistical analysis was calculated by SAS/ATAT NLIN (Version 8.0).

### 3. Results

#### 3.1. Resistance to flucarbazone-sodium

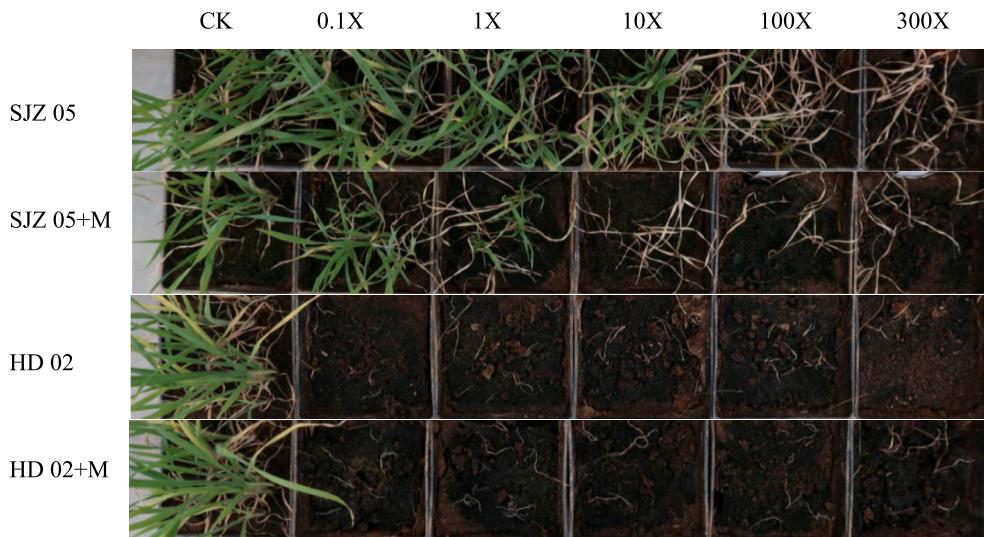
A total of 15 out of 44 Japanese brome populations were identified as resistant to flucarbazone-sodium (Table 2). The frequency of resistant populations was therefore 34% in Hebei province. The resistant populations were mainly distributed in Baoding and Shijiazhuang districts,

which both had 7 populations (BD 01–07 and SJZ 01–07). There was only one resistant population (LF 02) in Langfang district (Fig. 1). There were no resistant populations in Cangzhou, Hengshui, Xingtai and Handan districts. The resistance levels of the 15 resistant populations were different from each other. The highest resistance index was 1977 for SJZ 07, which was 46-fold more than the SJZ 05 population with a resistance index of 43. In addition, there were 6 populations where resistance indices were >1000, 7 populations were between 1000 and 100, and only 2 populations below 100 (Table 2).

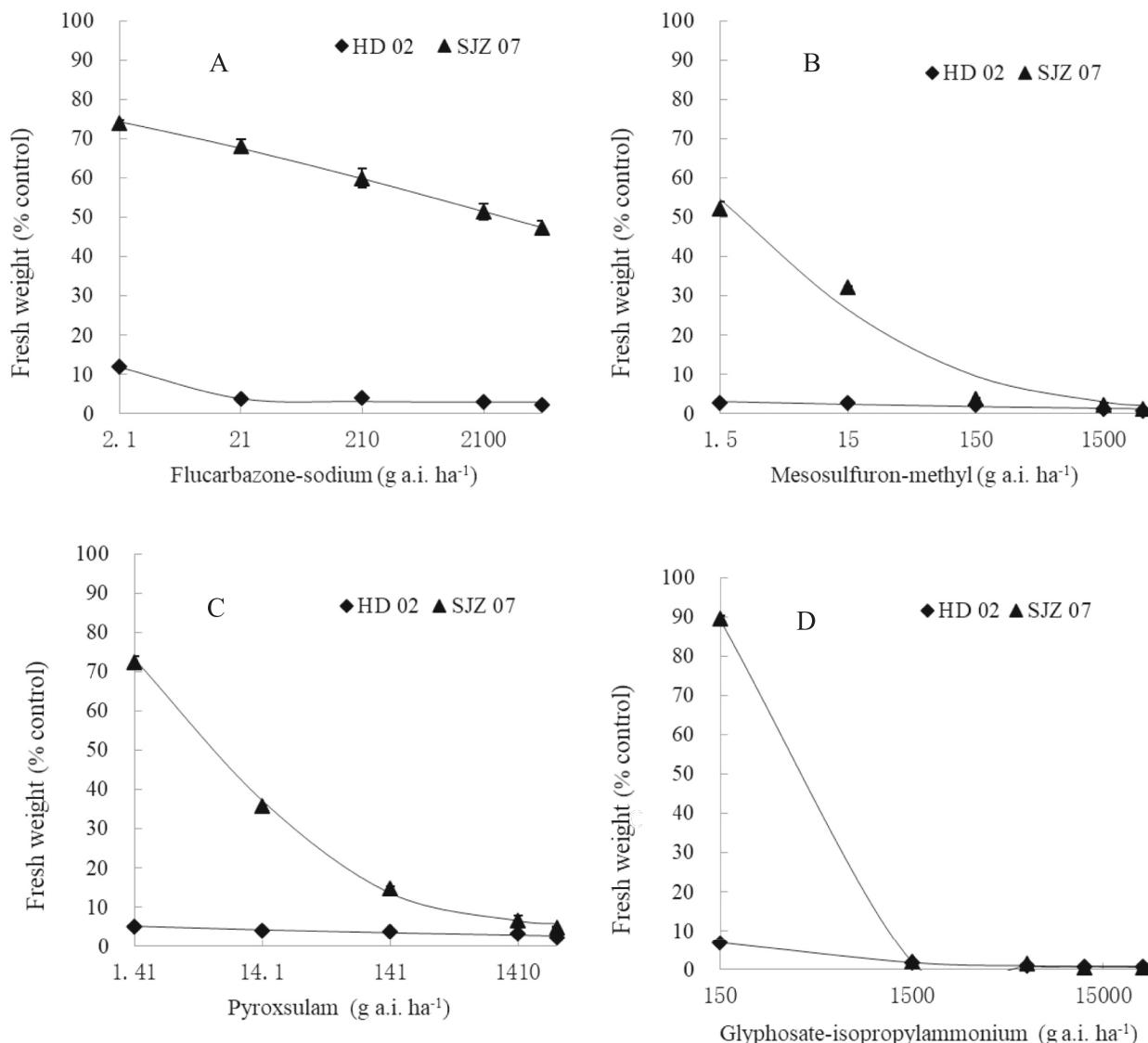
#### 3.2. ALS diversified mutations

The 1720 bp ALS sequences of three individuals of each Japanese brome population were aligned on NCBI database by Blastx and showed >96% homology with the ALS of *B. tectorum*. Amino acid mutations were found at Pro197 or Asp376 sites in resistant Japanese brome populations BD 01–07, SJZ 01–04 / 06–07, LF 02. However, there was no amino acid mutation identified in population SJZ 05 (Table 2).

ALS mutations were varied between the resistant populations. The Pro-197-Ser mutation was detected in BD 02–04, SJZ 01 / 03–04 / 06 and LF 02 populations in 3 plants. This mutation was present in 2 plants and Pro-197-Thr mutation was present in 1 plant in BD 01 and SJZ 02



**Fig. 3.** The response of SJZ 05 and HD 02 Japanese brome (*Bromus japonicus*) populations to flucarbazone-sodium without and with cytochrome P450 inhibitor malathion. M was cytochrome P450 inhibitor malathion at dose 2000 g a.i. ha<sup>-1</sup>, and X was flucarbazone - sodium at dose 21 g a.i. ha<sup>-1</sup>. The photos were 28 days after flucarbazone - sodium treatment.



**Fig. 4.** The fresh weight dose response curves of HD 02 and SJZ 07 Japanese brome (*Bromus japonicus*) populations to flucarbazone-sodium (A), mesosulfuron-methyl (B), pyroxslam (C) and glyphosate-isopropylammonium (D). Each value represented the mean of fresh weight (%control)  $\pm$  standard error.

populations. The Pro-197-Arg mutation was found in 1 plant in BD 05 and BD 06 populations, but the other two plants had no mutations in ALS. In the BD 07 population, 2 plants contained Pro-197-Thr and 1 plant contained Asp-376-Glu. All plants of SJZ 07 contained Pro-197-Thr. Pro-197-Ser was 68% of the mutations compared to Pro-197-Thr at 17%, Pro-197-Arg at 5% and Asp-376-Glu at 2% respectively (Table 2 and Fig. 2).

### 3.3. Metabolic resistance bioassay

The SJZ 05 population has no mutation in ALS, but a resistance index of 43. Pre-treatment of this population with P450 inhibitor malathion reduced the GR<sub>50</sub> for flucarbazone-sodium from 90.8 to 30.3 g a.i. ha<sup>-1</sup>. However, malation had no effect on flucarbazone-sodium activity against the susceptible population HD 02 (Table 2 and Fig. 3). This suggests that cytochrome P450 genes may be involved in resistance in this population.

**Table 3**

The cross - and multiple - resistance levels of SJZ 07 population.

Populations	Biotypes	Herbicides	GR <sub>50</sub> (g a.i. ha <sup>-1</sup> )	R/S
HD 02	S	Mesosulfuron - methyl	1.5	1
SJZ 07	R		2.3 $\pm$ 0.1	1.5
HD 02	S	Pyroxslam	1.41	1
SJZ 07	R		5.5 $\pm$ 0.1	3.9
HD 02	S	Glyphosate -	150	1
SJZ 07	R	isopropylammonium	324.6 $\pm$ 4.1	2.2

R was resistant biotype and S was susceptible biotype. GR<sub>50</sub> was the herbicide dose that induced 50% fresh weight reduction of Japanese brome (*Bromus japonicus*) populations. The R/S index was GR<sub>50</sub>(R)/GR<sub>50</sub>(S). When the doses of mesosulfuron - methyl, pyroxslam and glyphosate - isopropylammonium were at 1.5, 1.41 and 150 g a.i. ha<sup>-1</sup> respectively, the fresh weight inhibition of susceptible Japanese brome was over 50%, so 1.5, 1.41 and 150 g a.i. ha<sup>-1</sup> were selected as the GR<sub>50</sub> of susceptible population (Cui et al., 2011). Each GR<sub>50</sub> value represented the mean  $\pm$  standard error.

### 3.4. Cross - and multiple - resistance bioassay

The SJZ 07 population with Pro-197-Thr mutation had weak cross-resistance to mesosulfuron-methyl and pyroxsulam. When flucarbazone-sodium, mesosulfuron-methyl and pyroxsulam were used at the dose of 21, 15 and 14.1 g a.i. ha<sup>-1</sup> respectively, the fresh weight of SJZ 07 were 68%, 32% and 36% on the untreated (Fig. 4). The resistance index of SJZ 07 to mesosulfuron-methyl and pyroxsulam were 1.5 and 3.9 respectively (Table 3). In addition, this population had low level resistance to glyphosate with a resistance index of 2.2 (Fig. 4 and Table 3).

## 4. Discussion

With the change of farming practices, Japanese brome has become an important weed of wheat fields in Hebei province of China (Li et al., 2016; Xu et al., 2018). Flucarbazone-sodium has been widely used since first registered in China in 2008 (Xu et al., 2019). Two populations of Japanese brome resistant to flucarbazone-sodium were identified in 2022 in China (Lan et al., 2022; Li et al., 2022b). In this study, we found 15 populations resistant to flucarbazone-sodium, 6 > 1000-fold resistant and 7 populations with resistance indices from 100 to 1000. The distribution of resistant Japanese brome populations was regional, which were mainly distributed in Baoding and Shijiazhuang districts of Hebei province. Lan et al. also found a resistant Japanese brome population in Baoding district (Lan et al., 2022). Besides, so far only one resistant Japanese brome population was identified in Langfang district. There was no resistant Japanese brome population in Cangzhou, Hengshui, Xingtai and Handan districts. This indicates this weed will become more difficult to control in the future and more extensive surveys may identify further resistant populations.

ALS-inhibiting herbicides resistant weeds have diverse ALS mutations (Zhu et al., 2023). Mutations at positions 197 and 574 are the most commonly identified in ALS (Heap, 2023; Yu et al., 2010). In this study, Pro-197-Ser, Pro-197-Thr, Pro-197-Arg and Asp-376-Glu mutations were identified in Japanese brome. Most resistant plants carried the Pro-197-Ser mutation. Our results further demonstrated the diversified ALS mutations of resistant weeds.

Weed resistance can also be due to overexpression of the *ALS* gene (Gaines et al., 2020). Sen et al. (2021) reported that the *ALS* gene expression of resistant *B. sterilis* populations was greater than the susceptible populations (Sen et al., 2021). However, in this study, we did not detect a difference of *ALS* gene expression between resistant and susceptible Japanese brome populations.

In this study, the SJZ 07 population with Pro-197-Thr mutation had weak cross-resistance to mesosulfuron-methyl and pyroxsulam with resistance indices of 1.5 and 3.9. Li et al. (2022b) reported that a resistant Japanese brome population with Asp-376-Glu mutation evolved high cross-resistant to mesosulfuron-methyl and pyroxsulam with resistant indices of 44.3 and 37.8 respectively. Lan et al. (2022) also documented cross-resistance to these two herbicides in a Japanese brome population with Pro-197-Ser mutation (Lan et al., 2022). The cross-resistance levels of SJZ 07 population were lower than these previously reported populations, which shows that understanding the mutations present can identify herbicides that might still be active.

The SJZ 07 population also evolved weak resistance to glyphosate, which was unexpected. The resistant index was 2.2; however, the plants could be controlled by higher rates of glyphosate. Further research is required to understand how this population evolved resistance to glyphosate.

In one population without a target site mutation in ALS, flucarbazone-sodium resistance was reduced by the application of malathion, a CYP450 inhibitor, suggesting CYP450 could be involved in resistance. CYP450 have been found to be involved in many herbicide-resistant weeds, such as *D. sophia* (Yang et al., 2016), *C. bursa-pastoris* (Zhang et al., 2021), *M. aquaticum* (Bai et al., 2019), *L. rigidum* (Torra et al., 2021), *E. crus-galli* (Pan et al., 2022). Further research is required to

identify the specific enzyme involved in flucarbazone-sodium resistance in Japanese brome.

## 5. Conclusions

In conclusion, Japanese brome has evolved high resistance to flucarbazone - sodium, with 34% of populations in Hebei province of China with resistance. Resistant populations had diverse ALS mutations. Furthermore, CYP450 activity inhibition bioassay showed that CYP450 was involved in NTSR metabolic resistance in one Japanese brome population without an ALS mutation.

## Author contributions

Guichi Wang designed the experiments, and Silong Chen was responsible for data analysis. Xiaomin Liu, Bochui Zhao, Binghua Li, Beibei Shen, Zhizun Qi, Jianping Wang, Haiyan Cui participated the experiments. Xian Xu wrote the manuscript. All authors read and approved the manuscript.

## CRediT authorship contribution statement

**Xian Xu:** Writing – original draft, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Bochui Zhao:** Software, Investigation. **Binghua Li:** Resources, Methodology. **Beibei Shen:** Investigation. **Zhizun Qi:** Investigation. **Jianping Wang:** Investigation. **Haiyan Cui:** Investigation. **Silong Chen:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Funding acquisition. **Guichi Wang:** Funding acquisition. **Xiaomin Liu:** Funding acquisition.

## Declaration of competing interest

Authors declare no conflicts of interest for this study.

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## References

- Bai, S., Zhang, F.W., Li, Z.R., Wang, H.Z., Wang, Q., Wang, J.X., Liu, W.T., Bai, L.Y., 2019. Target-site and non-target-site-based resistance to tribenuron-methyl in multiply-resistant *Myosoton aquaticum* L. Pestic. Biochem. Physiol. 155, 8–14. <https://doi.org/10.1016/j.pestbp.2018.1012.1004>.
- Bai, S., Yin, M., Lyu, Q., Jiang, B., Li, L., 2022. Cytochrome P450 BsCYP99A44 and BsCYP704A177 confer metabolic resistance to ALS herbicides in *Beckmannia syzigachne*. Int. J. Mol. Sci. 23, 12175 doi. 12110.13390/ijms232012175.
- Baskin, J.M., Baskin, C.C., 1981. Ecology of germination and flowering in the weedy winter annual grass *Bromus japonicus*. J. Range Manag. 34, 369–372 doi. 310.2307/3897906.
- Dimaano, N.G., Iwakami, S., 2021. Cytochrome P450-mediated herbicide metabolism in plants: current understanding and prospects. Pest Manag. Sci. 77, 22–32 doi. 10.1002/ps.6040.
- Gaines, T.A., Duke, S.O., Morran, S., Rigon, C.A.G., Tranell, P.J., Kupper, A., Dayan, F.E., 2020. Mechanisms of evolved herbicide resistance. J. Biol. Chem. 295, 10307–10330 <https://www.jbc.org/cgi/doi/10310.11074/jbc.REV10120.013572>.
- Heap, I., 2023. The International Herbicide-Resistant Weed Database. Online. [www.weedscience.org](http://www.weedscience.org) (Accessed: October 9, 2023).
- Johnson, E.N., Wang, Z., Geddes, C.M., Coles, K., Hamman, B., Beres, B.L., 2018. Pyroxasulfone is effective for management of *Bromus* spp. in winter wheat in western Canada. Weed Technol. 32, 739–748. <https://doi.org/10.1017/wet.2018.70>.
- Lan, Y.N., Zhou, X.X., Lin, S.Y., Cao, Y., Wei, S.H., Huang, H.J., Li, W.Y., Huang, Z.F., 2022. Pro-197-Ser mutation and cytochrome P450-mediated metabolism conferring resistance to flucarbazone-sodium in *Bromus japonicus*. Plants 11, 1641. doi. 1610.3390/plants11131641.
- Li, J., Li, Y.B., Fang, F., Xue, D.X., Li, R.M., Gao, X.X., Li, M., 2022a. A novel naturally Phe206Tyr mutation confers tolerance to ALS-inhibiting herbicides in *Alopecurus*

- myosuroides*. Pestic. Biochem. Physiol. 186, 105156 <https://doi.org/10.1016/j.pestbp.2022.105156>.
- Li, Q., Du, L., Yuan, G.H., Guo, W.L., Li, W., Wang, J.X., 2016. Density effect and economic threshold of Japanese brome (*Bromus japonicus* Houtt.) in wheat. Chil. J. Agr. Res. 76, 441–447 doi: 410.4067/S0718-58392016000400007.
- Li, Q., Yu, J.P., Guo, W.L., Du, L., Bai, P.H., Liu, Y.X., 2022b. Target-site basis for resistance to flucarbazone-sodium in Japanese brome (*Bromus japonicus* Houtt.) in China. Chil. J. Agr. Res. 82, 493–501 doi: 410.4067/S0718-58392022000300493.
- Menegat, A., Kaiser, Y., Stephan, A., Ni, H.W., Gerhards, R., 2011. Chlorophyll fluorescence microscreening as a rapid detection method for herbicide resistance in grass weeds in North China plain winter wheat production systems and beyond. In: Proceedings of the 23rd Asian-Pacific Weed Science Society Conference, Cairns, QLD, Australia, 26–29 September.
- Metier, E.P., Lehnhoff, E.A., Mangold, J., Rinella, M.J., Rew, L.J., 2020. Control of downy brome (*Bromus tectorum*) and Japanese brome (*Bromus japonicus*) using glyphosate and four graminicides: effects of herbicide rate, plant size, species, and accession. Weed Technol. 34, 284–291 doi: 10.1017/wet.2019.112.
- Milani, A., Panizzo, S., Pinton, S., Danielis, R.A., Sattin, M., Scarabel, L., 2023. Diversity and spread of acetolactate synthase allelic variants at position 574 endowing resistance in *Amaranthus hybridus* in Italy. Plants 12, 332 doi: 310.3390/plants1202032.
- Ntoanidou, S., Madesis, P., Eleftherohorinos, I., 2019. Resistance of *Rapistrum rugosum* to tribenuron and imazamox due to Trp574 or Pro197 substitution in the acetolactate synthase. Pestic. Biochem. Physiol. 154, 1–6 doi: 10.1016/j.pestbp.2018.12.001.
- Pan, L., Guo, Q.S., Wang, J.Z., Shi, L., Yang, X., Zhou, Y.Y., Yu, Q., Bai, L.Y., 2022. CYP81A68 confers metabolic resistance to ALS and ACCase-inhibiting herbicides and its epigenetic regulation in *Echinochloa crus-galli*. J. Hazard. Mater. 428, 128225 doi: 128210.121016/j.jhazmat.122022.128225.
- Saleh, F.F., 2022. Responding wheat and weeds for seeding rates and herbicide as well its effect on yield and yield components. IOP Conf. Ser: Earth Environ. Sci. 1060.
- Sarani, A., Izadi-Darbandi, E., Rastgoo, M., Galavi, M., 2021. Optimizing of sulfosulfuron and sulfosulfuron plus metsulfuron-methyl herbicides efficiency against Japanese downy brome *Bromus japonicus* L. by adjuvants application. J. Crop Prot. 10, 85–94.
- Sen, M.K., Hamouzová, K., Mikulka, J., Bharati, R., Košnarová, P., Hamouz, P., Roy, A., Soukup, J., 2021. Enhanced metabolism and target gene overexpression confer resistance against acetolactate synthase-inhibiting herbicides in *Bromus sterilis*. Pest Manag. Sci. 77, 2122–2128 doi: 2110.1002/ps.6241.
- Torra, J., Montull, J.M., Taberner, A., Onkokesung, N., Boonham, N., Edwards, R., 2021. Target-site and non-target-site resistance mechanisms confer multiple and cross-resistance to ALS and ACCase inhibiting herbicides in *Lolium rigidum* from Spain. Front. Plant Sci. 12, 625138 doi:625110.623389/fpls.622021.625138.
- Wang, M.F., 1986. Occurrence, spread and control of Japanese brome in the wheat. Weed Sci. 2, 3–5.
- Xu, X., Liu, G.Q., Chen, S.L., Li, B.H., Liu, X.M., Wang, X.Y., Fan, C.Q., Wang, G.Q., Ni, H.W., 2015. Mutation at residue 376 of ALS confers tribenuron-methyl resistance in flixweed (*Descurainia sophia*) populations from Hebei province, China. Pestic. Biochem. Physiol. 125, 62–68. <https://doi.org/10.1016/j.pestbp.2015.05.008>.
- Xu, X., Liu, X.M., Li, B.H., Shen, B.B., Wang, J.P., Cui, H.Y., Qi, Z.Z., Tian, S.M., Wang, G.Q., 2018. Composition of weed communities in winter wheat middle and south of Hebei province. J. Weed Sci. 36, 6–12. <https://doi.org/10.19588/j.issn.11003-19935X.12018.19504.19002>.
- Xu, X., Liu, X.M., Li, B.H., Shen, B.B., Wang, J.P., Cui, H.Y., Qi, Z.Z., Tian, S.M., Wang, G.Q., 2019. Control effect of six herbicides on *Aegilops tauschii* and *Bromus japonicus*. J. Weed Sci. 37, 42–46. <https://doi.org/10.19588/j.issn.11003-19935X.12019.19503.19008>.
- Yang, Q., Deng, W., Li, X.F., Yu, Q., Bai, L.Y., Zheng, M.Q., 2016. Target-site and non-target-site based resistance to the herbicide tribenuron-methyl in flixweed (*Descurainia sophia* L.). BMC Genomics 17, 551 doi: 510.1186/s12864-12016-12915-12868.
- Yu, Q., Powles, S.B., 2014. Resistance to AHAS inhibitor herbicides: current understanding. Pest Manag. Sci. 70, 1340–1350. <https://doi.org/10.1002/ps.3710>.
- Yu, Q., Han, H.P., Vila-Aiub, M.M., Powles, S.B., 2010. AHAS herbicide resistance endowing mutations: effect on AHAS functionality and plant growth. J. Exp. Bot. 61, 3925–3934 doi:3910.1093/jxb/erq3205.
- Zhang, L.L., Guo, W.L., Li, Q., Wu, C.X., Zhao, N., Liu, W.T., Wang, J.X., 2017. Tribenuron-methyl resistance and mutation diversity of the AHAS gene in shepherd's purse (*Capsella bursa-pastoris* (L.) Medik.) in Henan Province, China. Pestic. Biochem. Physiol. 143, 239–245 doi:210.1016/j.pestbp.2017.1005.1007.
- Zhang, X.L., Wang, H.Z., Bei, F., Wu, C.X., Zhang, L.L., Jia, S.S., Wang, J.X., Liu, W.T., 2021. Investigating the mechanism of metabolic resistance to tribenuron-methyl in *Capsella bursa-pastoris* (L.) Medik. By full-length transcriptome assembly combined with RNA-Seq. J. Agric. Food Chem. 69, 3692–3701 doi:3610.1021/acs.jafc.3690c07512.
- Zhu, G.T., Wang, H., Gao, H.T., Liu, Y., Li, J., Feng, Z.K., Dong, L.Y., 2023. Multiple resistance to three modes of action of herbicides in a single Italian ryegrass (*Lolium multiflorum* L.) population in China. Agronomy 13, 216 doi:210.3390/agronomy13010216.
- Zou, Y.Z., Zhao, B.C., Cao, S.Y., Guan, Y.D., Liu, L.R., Ji, M.S., 2022. Mutation at the 197 site and P450-mediated metabolic resistance are involved in bensulfuron-methyl resistance in *Sagittaria trifolia*. Plant Sci. 331, 111700. <https://doi.org/10.111016/j.plantsci.112023.111700>. Epub 112023 Apr 111706.