

Target-site basis for resistance to flucarbazone-sodium in Japanese brome (*Bromus japonicus* Houtt.) in China

Qi Li¹, Jinping Yu¹, Wenlei Guo², Long Du³, Penghua Bai¹, and Yixue Liu^{1*}

¹Institute of Plant Protection, Tianjin Academy of Agricultural Sciences, Tianjin 300381, PR China.

*Corresponding author (liuyixue430@163.com).

²Plant Protection Research Institute, Guangdong Academy of Agricultural Sciences/Guangdong Provincial Key Laboratory of High Technology for Plant Protection, Guangzhou 510640, PR China.

³Pest Bio-control Lab, Shandong Peanut Research Institute, Qingdao 266041, PR China.

Received: 18 January 2022; Accepted: 24 April 2022; doi:10.4067/S0718-58392022000300493

ABSTRACT

Japanese brome (*Bromus japonicus* Houtt.) is a troublesome annual weed and widely distributed in winter wheat (*Triticum aestivum* L.) fields in the North China Plain. A *B. japonicus* population (TJ06) suspected of resistance to acetolactate synthase (ALS) inhibitors was found in Tianjin, China. In this study, the TJ06 population with an Asp-376-Glu mutation in ALS gene was identified. TJ06 population developed 66.7-fold resistance to flucarbazone-sodium and exhibited obvious cross-resistance to other two ALS-inhibiting herbicides. The 50% plant growth reduction (GR₅₀) to herbicides of pyroxsulam and mesosulfuron-methyl were 28.72 and 39.44 g ai ha⁻¹, respectively. In in vitro ALS activity assays, the concentration of flucarbazone-sodium required to inhibit 50% ALS activity (I₅₀) for TJ06 was 11.3-fold greater than that for a susceptible population (TJ01), which was highly correlated with that of whole-plant response experiments and indicated that the Asp-376-Glu mutation leading to resistance reduced sensitivity of the ALS enzyme to flucarbazone-sodium. Besides, one derived cleaved amplified polymorphic sequence (dCAPS) marker was designed to quickly detect Asp376 mutation in ALS gene of *B. japonicus*.

Key words: ALS, *Bromus japonicus*, dCAPS, gene mutation, herbicide resistance.

INTRODUCTION

Japanese brome (*Bromus japonicus* Houtt.) is a winter annual weed belonging to the brome family. Generally, seedlings appear in September and October, flower in early May and seed dispersal begins in early October (Baskin and Baskin, 1981). A *B. japonicus* plant can produce an average of 1885 seeds dispersed by water or wind because of their light weight, which can germinate over a wide temperature range of 5 to 30 °C without light under a large scale of pH (Li et al., 2015). *Bromus japonicus* competes with wheat for nutrients, light, water, and space, which may reduce yield by at least 30% in heavily infested fields (Vigueira et al., 2013; Li et al., 2015). In China, the economic threshold for *B. japonicus* in wheat was between 4 and 5 plants m⁻² with 80% efficiency for the herbicide flucarbazone, suggesting that even low plant populations may reduce significantly crop yields (Li et al., 2016). With the changes of farming systems and long-term use of herbicides, *B. japonicus* has become one of the most notorious weeds in the wheat fields of China.

Postemergence treatment of flucarbazone-sodium has been registered in China since 2008 and used more than 10 yr in wheat fields. Flucarbazone-sodium (here after delineated flucarbazone) is an acetolactate synthase (ALS) inhibitor especially used for controlling *B. japonicus* in China. Zhao et al. (2017b) tested 13 herbicides by dose-response experiments in glasshouses and field experiments and found that flucarbazone showed the best control efficacy against

B. japonicus. Unfortunately, in recent years, farmers from Tianjin City, China, have observed that flucarbazone failed to control *B. japonicus* in wheat fields at the recommended rate (31.5 g ai ha⁻¹).

Acetolactate synthase is a critical enzyme in the biosynthetic pathway of the branched-chain amino acids, isoleucine, valine, and leucine (Yu and Powles, 2014; Walter et al., 2014; Liu et al., 2015). ALS-inhibiting herbicides inhibit the formation of acetoxybutyrate and acetolactate, cause starvation of branched-chain amino acid and lead to the death of susceptible plants (Powles and Yu, 2010; Dayan et al., 2015). ALS-inhibiting herbicides have five groups including imidazolinone (IMI), triazolopyrimidine (TP), pyrimidinyl thiobenzoate (PTB), sulfonylurea (SU) and sulfonylaminocarbonyl triazolinone (SCT) (Yu and Powles, 2014). Because of high efficacy, broad-spectrum weed control, favorable environmental profile, high level of crop safety and low mammalian toxicity, ALS-inhibiting herbicides have been widely used worldwide (Zhao et al., 2017a). However, the over-reliance on the same mode of action herbicides will inevitably result in the evolution of herbicide resistance quickly (Neve et al., 2014). Due to the extensive and persistent use in the past few decades, 166 weed species have evolved resistance to ALS inhibitors (Heap, 2021).

Two mechanisms, target-site-based resistance (TSR) and non-target-site-based resistance (NTSR), are responsible for weed resistance evolution (Délye, 2013; Liu et al., 2015). In most cases, the resistance to ALS-inhibiting herbicides is caused by mutations in the ALS gene (Yu and Powles, 2014). To date, 32 resistance conferring mutations have been identified at eight sites (Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653, Gly654) in more than 50 weed species (Xu et al., 2020). Cross-resistance is commonly observed in resistant weed species (Yu and Powles, 2014; Tranel et al., 2022). The TSR conferred by mutations in the ALS gene may reduce the sensitivity of target enzymes to special herbicides or lead the target gene to overexpression (Ntoanidou et al., 2017; Zhao et al., 2018). So, TSR was confirmed to be responsible for most cases of cross-resistance to ALS inhibiting herbicides (Yu et al., 2008). Generally, mutations at Trp574 endow resistance to SU, TP, and IMI herbicides, whereas mutations at Ala205 endow resistance to all five groups (Tranel et al., 2022).

In July 2018, a *B. japonicus* population (TJ06) that failed to be controlled by flucarbazone at the field-recommended rate was found in a winter wheat field in Tianjin City, China. This study aimed (1) to identify the resistant level of TJ06 to flucarbazone; (2) to investigate the molecular basis of resistance; (3) to evaluate the pattern of cross-resistance to ALS-inhibiting herbicides for *B. japonicus* population with specific ALS mutations.

MATERIALS AND METHODS

This research was carried out in 2020, Tianjin Academy of Agricultural Sciences, Institute of Plant Protection, Weed Science Laboratory (39°6'14" N, 117°3'32" E; 3 m a.s.l.), in Tianjin, China. The research was conducted in North China Plain, in this region wheat (*Triticum aestivum* L.) is one of the main crops and Japanese brome (*Bromus japonicus* Hoult.) has caused severe losses to wheat production due to resistance to herbicides. For these reasons, two *B. japonicus* populations were used in the study.

Plant materials

Seeds of putative resistant population (TJ06) were collected from wheat fields in 2018 at Tianjin city, China, where flucarbazone had failed to control this weed. The susceptible population (TJ01) was collected from non-cultivated lands never treated with herbicides (Table 1). Seeds of each population were randomly collected from at least 30 mature plants. After collection, seeds were stored in paper bags at 4 °C until used in experiments.

Table 1. Location at which the *Bromus japonicus* seeds were collected and the associated herbicide application history.

Population	Biotype susceptibility	Location				GPS coordinates	Herbicide application history
		Country	District	City	Site		
TJ01	Susceptible	Kangningli	Xiqing	Tianjin	Uncultivated land	39°6'14" N, 117°3'32" E	Never applied
TJ06	Resistant	Zoujiazui	Jinghai	Tianjin	Wheat field	38°48'33" N, 116°55'6" E	Flucarbazone ≥ 10 yr

*Nucleotide introduced to determine a restriction site is underlined.
dCAPS: Derived cleaved amplified polymorphic sequence assay.

The seeds were sown on 9 cm petri dishes lined with two layers of Whatman N°1 filter paper (Cytiva, Marlborough, Massachusetts, USA) moistened with 5 mL distilled water. The dishes were kept in controlled-environment growth chambers at 25 °C and 12 h photoperiod (Model RXZ, Ningbojiangnan Instrument Factory, Ningbo, China). After 4 d, 15 germinated seeds were transplanted into a 11 cm × 9 cm plastic pots filled with loam soil. The soil was passed through a 3 mm sieve with 1.7% organic matter. The pots were watered as needed and transferred in a greenhouse with day/night temperatures set at 25 ± 5/20 ± 5 °C with a 12 h photoperiod. The *B. japonicus* seedlings were thinned to nine evenly sized plants before herbicide application.

Single-dose herbicide resistance testing

Seedlings at the 3- to 4-leaf stage were treated with flucarbazone (70% WDG, Arysta Life Science (Shanghai) Co., Ltd., Shanghai, China) at the recommended field application rate (31.5 g ai ha⁻¹) using a laboratory sprayer (Wang et al., 2019). Third untreated pots of each population were treated with water as the control. The surviving plants (plants with new green leaves were considered alive) were assessed at 21 d after treatment (DAT). All treatments were replicated three times and the experiment was conducted twice.

Amplification and sequencing of the ALS gene

Approximately 100 mg young leaf tissues were harvested from each *B. japonicus* plant at the 3- to 4-leaf stage for genomic DNA extraction by EasyPure Plant Genomic DNA Kit (TransGen Biotech, Beijing, China). One primer pair (Table 2) was designed to amplify the ALS gene containing eight resistance-endowing amino acid residues by Primer Premier v6.0 based on the homologous sequences of ALS genes from *Bromus tectorum* (AF488771.1).

PCR amplifications were conducted in a total volume of 25 µL per sample, which contained 30 ng template DNA, 1 µL each primer (10 µM), 12.5 µL 10×EasyTaq SuperMix (TransGen Biotech) and 9.5 µL nuclease-free water. PCR was performed on a thermal cycler (T100, BIO-RAD, Hercules, California, USA) programmed using a program of one cycle of 95 °C for 4 min, followed by 36 cycles of 95 °C for 20 s, 58 °C for 30 s, 72 °C for 2 min, with a final elongation at 72 °C for 10 min.

The amplification products were subjected to electrophoresis on 1.5% agarose gel buffered with 1× TAE, and purified using an EasyPure Quick Gel Extraction Kit (TransGen Biotech). The purified PCR products were cloned into the pEASY-T1 Cloning Kit (TransGen Biotech) and transferred into Trans1-T1 Phage Resistant Chemically Competent Cells (TransGen Biotech). Next, 10 positive clones from each individual plant were sequenced by GENEWIZ (South Plainfield, New Jersey, USA). The ALS gene of *B. japonicus* was aligned and compared using the DNAMAN v.6.0.3 software (Lynnon Biosoft, Quebec, Canada).

Whole-plant dose-response experiment

Seeds of TJ01 and TJ06 populations were planted as described above. At the 3- to 4- leaf stage, herbicides were sprayed at a series of doses (Table 3) as described above. The above-ground shoots were harvested 21 DAT and the dry weights were recorded. All treatments were replicated three times and the experiment was conducted twice.

Table 2. Primers used in this study.

Name	Sequence 5'-3' ^a	Usage	Covering the confirmed point mutations	Recognition site	Restriction enzyme	Product size (bp)
Forward	ATCGTCGACGTCTTCGCCTACCC	Sequencing	Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653, Gly654	-	-	1724
Reverse	TTATCCCTACAAAGCGAACA					
d376-F	CTGCTTGCGTTTGGTGTGCGATTGACGA	dCAPS	-	CGAT▼CG	<i>PvuI</i>	237
d376-R	CTCCAACCTCCTCGCCATGGA					

^aNucleotide introduced to determine a restriction site is underlined.

dCAPS: Derived cleaved amplified polymorphic sequence assay.

Table 3. Herbicide treatments applied in the whole-plant experiment.

Herbicide ^a	TJ01 (S)	TJ06 (R)
	g ai ha ⁻¹	
Flucarbazone	0.12, 0.49, 1.97, 7.87, 31.5, 126	1.97, 7.87, 31.5, 126, 504, 2016
Pyroxsulam	0.13, 0.39, 1.17, 3.52, 10.57, 31.7	1.17, 3.52, 10.57, 31.7, 95.1, 285.3
Mesosulfuron-methyl	0.11, 0.33, 1, 3, 9, 27	3, 9, 27, 81, 243, 721

^aFlucarbazone (70% WDG, Arysta Life Science (Shanghai) Co., Ltd., China), Pyroxsulam (4% OD, Kedihua Agricultural Technology, China), Mesosulfuron-methyl (30 g L⁻¹ OD, Bayer (China) Limited, China).
S: Susceptible to acetolactate synthase (ALS) inhibitors; R: resistant to ALS inhibitors.

In vitro ALS activity assay

Seedlings at 3- to 4-leaf stage were used for ALS activity assay in vitro. The ALS extraction and ALS activity assay were performed according to Yu et al. (2010) and Han et al. (2012a). ALS activity in vitro was implemented colorimetrically (530 nm) using a microplate reader (SpectraMax, Molecular Devices, San Jose, California, USA) by measuring the acetoin production. All treatments were replicated three times and the experiment was conducted twice with independent enzyme extracts.

The active-ingredient concentrations of commercial formulation flucarbazone used in ALS activity assays were: 0.0, 0.000765, 0.00383, 0.0191, 0.0956, 0.478, 2.39, 11.9, and 59.8 µM for the TJ01 population and 0.0, 1.86, 3.73, 7.47, 14.9, 29.9, 59.8, 119.5, and 239.1 µM for the TJ06 population.

Derived cleaved amplified polymorphic sequence (dCAPS) assay for detection of Asp-376-Glu mutations

A dCAPS assay including three-step (PCR, restriction digestion, and gel electrophoresis) was designed to detect the single nucleotide polymorphisms (SNP) at codon 376 of ALS. According to the predetermined sequence information, the Asp to Glu substitution was caused by a nucleotide substitution at the third position in codon 376 of *B. japonicus* (GAT to GAG).

One pair of primers and corresponding restriction enzyme (Table 2) were chosen using the dCAPS Finder 2.0 program (Guo et al., 2015). A mismatch was introduced in the primer d376-F to create a restriction site CGAT▼CG for the restriction enzyme *PvuI* in the susceptible sequence. Primer d376-F enforced the second base of 376 codon and any changes in the third base would disrupt the restriction site.

Total genomic DNA extraction and PCR amplifications were as described above. Digestions were performed at 37 °C for 20 min using 4 µL PCR product incubated with 1 µL (10 U) restriction enzyme *PvuI*, 2 µL enzyme buffer and 13 µL ddH₂O. After digestion, dCAPS products were visualized on 3% agarose gel electrophoresis in 1 × TAE buffer at 140 V for 0.5 h.

Statistical analyses

Data from the repeated experiments were analyzed by ANOVA using SPSS V.22.0 software (IBM Corporation, Armonk, New York, USA) and there was nonsignificant ($P < 0.05$) trial-by-treatment interaction between the repeated experiments.

The regression analyses in dose-response experiments and ALS activity assays were conducted using SigmaPlot v.13.0 software (Systat Software, San Jose, California, USA). Herbicide dose causing 50% growth reduction (GR₅₀) or 50% inhibition of ALS activity (I₅₀) were evaluated by a four-parameter log-logistic equation (Cao et al., 2021):

$$y = c + (d - c) / [1 + (x / GR_{50} \text{ or } I_{50})^b]$$

where c is the lower limit, d is the upper limit, b is the slope in GR₅₀ or I₅₀, x is the independent variable (herbicide dose), y is the dependent variable (percentage of dry weight or ALS activity).

The fitted equation was used to evaluate the GR₅₀ and I₅₀. The resistance index (RI) was the ratio calculated by the GR₅₀ or I₅₀ of resistant population divided by that of susceptible population.

RESULTS

Sensitivity to flucarbazone and other herbicides

In single-dose testing, all plants of TJ01 population died at 31.5 g ai ha⁻¹ of flucarbazone, whereas all plants of TJ06 population survived. In dose-response bioassays (Table 4, Figure 1a), the TJ06 population were 66.7-fold more resistant than the TJ01 population based on RI value, which indicated that TJ06 population had evolved extremely high resistance to flucarbazone.

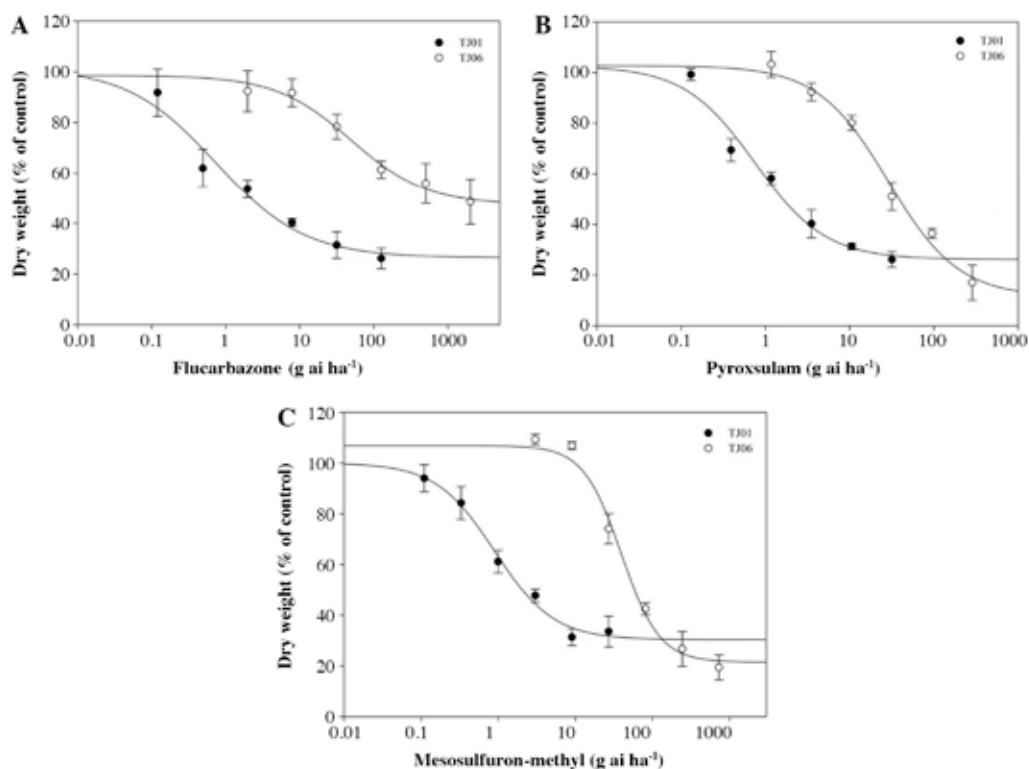
Table 4. GR₅₀ values of *Bromus japonicus* populations treated with acetolactate synthase (ALS) inhibitors and alternative herbicides and resistance index (RI) indicated by R/S ratios.

Herbicide	TJ01 (S)	TJ06 (R)	RI
	g ai ha ⁻¹		
Flucarbazone	0.74 ± 0.35b	49.36 ± 16.27a	66.7
Pyroxsulam	0.76 ± 0.27b	28.72 ± 8.57a	37.8
Mesosulfuron-methyl	0.89 ± 0.17b	39.44 ± 7.88a	44.3

Each value represents the mean ± standard error. Data points without our overlapping error bars are considered significantly different.

GR₅₀: Herbicide dose causing a 50% fresh-weight growth reduction; S: susceptible to ALS inhibitors; R: resistant to ALS inhibitors.

Figure 1. Dose response (percentages of control) of susceptible (TJ01, ●), and resistant (TJ06, ○) *Bromus japonicus* populations to different herbicides: flucarbazone (a), pyroxsulam (b), and mesosulfuron-methyl (c).



Data represent the mean ± SE of two experiments, each comprising three replicates. Data points without our overlapping error bars are considered significantly different.

The resistance pattern to other herbicides was also determined in this study. The results showed that the TJ06 population displayed different resistant levels to other ALS-inhibiting herbicides: pyroxsulam and mesosulfuron-methyl (Table 4, Figure 1). As shown in Table 4, the GR₅₀ values of pyroxsulam and mesosulfuron-methyl were 0.76 to 0.89 g ai ha⁻¹ for the TJ01 population, and were 28.72 to 39.44 g ai ha⁻¹ for the TJ06 population. Based on the RI values, the TJ06 population is 37.8- and 44.3-fold resistant to pyroxsulam and mesosulfuron-methyl, respectively.

Identification of ALS gene resistance mutation in *B. japonicus*

In this study, 10 plants were randomly selected and ALS gene sequenced from each population. A 1724-bp ALS gene fragment that included five conserved domains was amplified from each plant. The sequences from different populations showed 94.59% identity with the documented ALS gene from *B. tectorum*, indicating that the target genes were amplified.

Most of these single nucleotide polymorphisms (SNPs) were synonymous without resulting in any amino acid substitution. No mutation conferring resistance to ALS inhibitors was detected in the plants of TJ01 population. However, a thymine-to-guanine mutation was detected in all 10 plants of TJ06 population leading to a substitution of Asp (GAT) to Glu (GAG) at codon 376 of ALS. In addition, no difference was found between the S and R populations at nucleotide sequence positions 122, 197, 205, 377, 574, 653 and 654, where point mutations causing resistance have been reported in other weed species.

Inhibition of *B. japonicus* ALS activity by flucarbazone

The in vitro ALS activity assays indicated that the total ALS activity between TJ01 and TJ06 populations was similar. The specific activities were 16.7 ± 0.16 and 15.2 ± 0.23 nmol acetoin mg^{-1} protein min^{-1} for TJ01 and TJ06, respectively (Table 5). However, in the presence of flucarbazone, the ALS extracted from TJ06 plants showed less sensitivity than that from TJ01 plants (Figure 2). As shown in Table 5, the I_{50} values of TJ01 and TJ06 were 1.55 and 17.52 μM , respectively. The I_{50} value for the R population (TJ06) was 11.3-fold greater than that for the S population (TJ01). These results demonstrated that an insensitive ALS was likely responsible for conferring resistance to flucarbazone in the R populations.

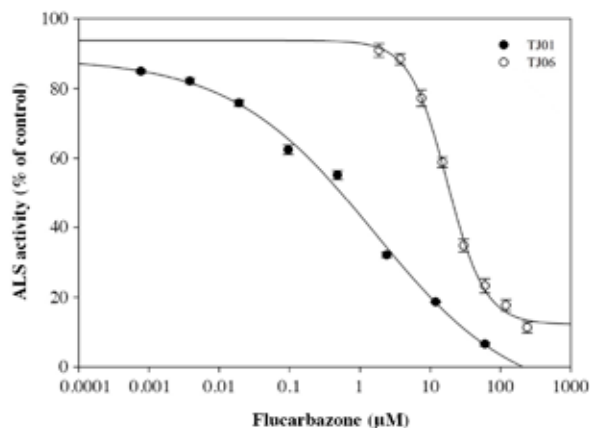
The dCAPS assay realized a quick determination of the allelic variation at the specific mutation site. The primer pair d376-F and d376-R amplified a 237 bp DNA fragment. After restriction with *PvuII*, the wild type plants displayed a 207 bp digested band (with a 30 bp fragment invisible on the gel), while the homozygous mutant type plants showed a 237 bp undigested fragment for losing the recognition site at the third base of codon 376 of ALS (T to G mutation). Heterozygous plants displayed both the wild 207 bp and mutated 237 bp DNA fragments (Figure 3).

Table 5. Total acetolactate synthase (ALS) activity without flucarbazone and the I_{50} and resistance ratio (R/S) values determined with a partially purified ALS enzyme isolated from susceptible (S) and resistant (R) *Bromus japonicus* populations.

Population	State	Total ALS activity	R/S	I_{50}	R/S
		nmol acetoin mg^{-1} protein min^{-1}		μM	
TJ01	S	$16.7 \pm 0.16a$		$1.55 \pm 0.92b$	
TJ06	R	$15.2 \pm 0.23b$	0.91	$17.52 \pm 1.07a$	11.3

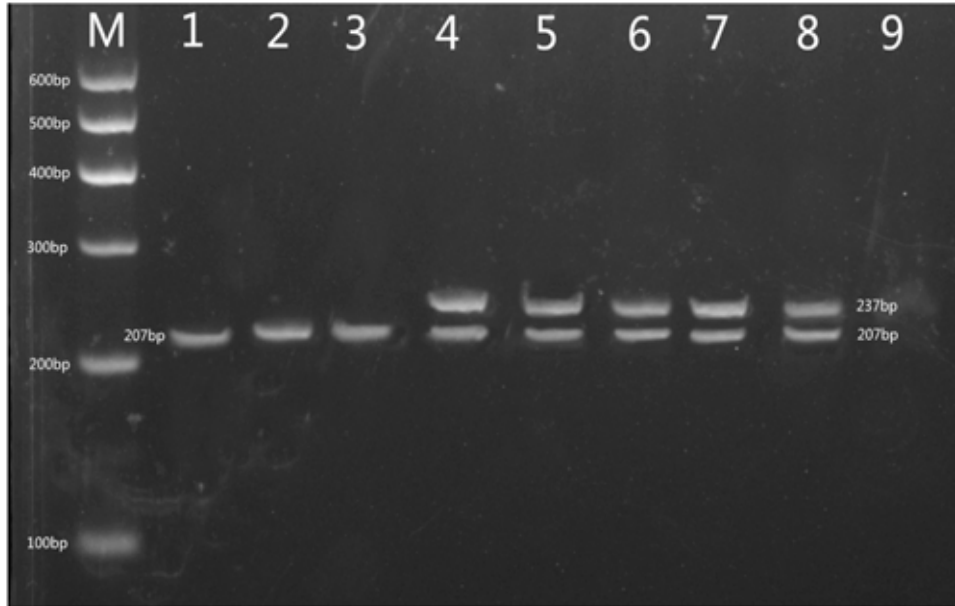
The values represent the mean (\pm SE) of two experiments, each containing three replicates. Different letters indicate significant differences ($P \leq 0.05$ level) according to Tukey's honest significant difference (HSD) test. I_{50} : Herbicide concentration required to inhibit ALS activity by 50% compared with the untreated control. R/S was calculated by dividing the I_{50} value of the resistant population by that of the susceptible population.

Figure 2. Inhibition of acetolactate synthase (ALS) activities in populations susceptible (TJ01) and resistant (TJ06) to flucarbazone.



Data represent the mean \pm SE of two extractions, each comprising three replicates. Data points without our overlapping error bars are considered significantly different.

Figure 3. Derived cleaved amplified polymorphic sequence (dCAPS) assay of individual *Bromus japonicus* plants. *PvuI* digestion profile for 376 mutation of acetolactate synthase (ALS).



M: DNA marker; lane 1-3: wild type plants from the TJ01 population; lanes 4-8: heterozygous resistant plants from the TJ06 population; lane 9: H₂O PCR control (no DNA).

DISCUSSION

Since flucarbazone was introduced into China, many Chinese farmers have used it repeatedly as the single method for *B. japonicus* control by postemergence treatment. In addition, farmers were gradually increasing herbicide dosage for ensuring the control efficacy. After 3 to 5 yr of persistent and intensive application, the ALS inhibitors would result in rapid evolution of herbicide resistance (Powles and Yu, 2010). *Bromus japonicus* was relied extensively on ALS inhibitors for control. Flucarbazone, mesosulfuron-methyl, pyroxsulam have been continuously applied for many years in China. In this study, TJ06 population has evolved extremely high resistance to flucarbazone. The mutation in ALS gene has been confirmed to be the most commonly mechanism for the resistance to ALS inhibitors in many weeds. Amino acid substitution at Asp-376 (Asp-376-Glu) was found in surviving plants of TJ06 population, and same mutation was reported in several weeds such as Powell's amaranth (*Amaranthus powellii*), horseweed (*Conyza canadensis*), wild radish (*Raphanus raphanistrum*), rock bulrush (*Schoenoplectus juncooides*) (Heap, 2021). The result indicates that Asp-376-Glu mutation was responsible for the resistance to ALS inhibitors in *B. japonicus*. This is the first report of the Asp-376-Glu mutation in *B. japonicus* populations.

According to reports, many weeds evolving resistance to ALS inhibitors exhibited increased ALS activity compared to susceptible populations (Yu et al., 2010). However, this result of the in vitro ALS activity assay indicated no difference between S and R populations. It is similar to the *Descurainia sophia* and *Myosoton aquaticum* (Han et al., 2012b; Liu et al., 2013). In most cases, reducing target-enzyme sensitivity to the herbicide could endow resistance to weeds (Liu et al., 2013). In this study, the ALS sensitivity to flucarbazone of R plants (TJ06) was significantly reduced, which may result in the high resistance.

Up to now, only one amino acid substitution (Asp to Glu) endowing herbicide resistance has been identified in codon 376 of ALS (Yu and Powles, 2014). The dCAPS assay designed by authors in this research could quickly identify the homo/heterozygous status of the plants at Asp376 amino acid residues without the gene sequencing. According to the results, the intensity of the amplification product was always sufficient to be visible on agarose gels.

In this study, the resistant plants of TJ06 population always have the Asp-376-Glu mutation. However, the non-target-site-based resistance (NTSR) could not be absolutely excluded out and might be possibly involved in conferring herbicide resistance. One resistant weed population may simultaneously contain target-site-based resistance (TSR) and NTSR (Ntoanidou et al., 2019). Moreover, NTSR usually conferred lower resistance on weed than TSR (Li et al., 2019). So, the high resistance of *B. japonicus* population was caused by TSR, at least partly. In the future, more work is needed to explain whether NTSR was involved in the resistant populations.

The cross-resistance was commonly observed in weed species evolving resistance to ALS inhibitor and depends on many factors, such as weed species, substitution position, specific mutation amino acids and ALS inhibitor type (Yu and Powles, 2014). Up to now, 12 weeds have evolved high resistance to ALS inhibitor with the Asp-376-Glu mutation. But the cross-resistance patterns conferred by Asp-376-Glu mutation are not the same (Yuan et al., 2021). For example, the *A. powellii* with an Asp-376-Glu mutation exhibited high resistance to all five ALS-inhibiting herbicides, however, the horseweed exhibited resistance to sulfonylurea (SU), pyrimidinyl thiobenzoate (PTB), triazolopyrimidine (TP) and imidazolinone (IMI) (Ashigh et al., 2009; Zheng et al., 2011). In this study, R population of *B. japonicus* exhibited relatively high-level resistance to mesosulfuron-methyl (SU), pyroxsulam (TP) and flucarbazone (SCT) herbicides. Determining the cross-resistance to ALS inhibitor in *B. japonicus* is significant for farmers to select alternative herbicides for controlling this weed. Furthermore, farmers should use herbicides with different modes of action in rotation and adopt more integrated control strategies to delay evolution of resistance.

CONCLUSIONS

In summary, this study confirms that TJ06 population of *Bromus japonicus* has evolved high-level resistance to flucarbazone, and demonstrates the molecular mechanism underlying this resistance by an Asp-376-Glu mutation in the ALS gene. These results demonstrate that persistent and intensive application of flucarbazone-sodium has resulted in resistance in *B. japonicus* in China, and an integrated weed management system is highly necessary for *B. japonicus* control in wheat fields.

ACKNOWLEDGEMENTS

This work was supported by the Creative Research for Young Scientists of Tianjin Academy of Agricultural Sciences (China) (Nr 2021017).

REFERENCES

- Ashigh, J., Corbett, C.L., Smith, P.J., Laplante, J., and Tardif, F.J. 2009. Characterization and diagnostic tests of resistance to acetohydroxyacid synthase inhibitors due to an Asp₃₇₆Glu substitution in *Amaranthus powellii*. *Pesticide Biochemistry and Physiology* 95:38-46.
- Baskin, J.M., and Baskin, C.C. 1981. Ecology of germination and flowering in the weedy winter annual grass *Bromus japonicus*. *Journal of Range Management* 34:369-372.
- Cao, Y., Wei, S., Huang, H., Li, W., Zhang, C., and Huang, Z. 2021. Target-site mutation and enhanced metabolism confer resistance to thifensulfuron-methyl in a multiple-resistant redroot pigweed (*Amaranthus retroflexus*) population. *Weed Science* 69:161-166.
- Dayan, F.E., Owens, D.K., Corniani, N., Silva, F.M.L., Watson, S.B., Howell, J.L., et al. 2015. Biochemical markers and enzyme assays for herbicide mode of action and resistance studies. *Weed Science* 63:23-63.
- Délye, C. 2013. Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade. *Pest Management Science* 69:176-187.
- Guo, W.L., Yuan, G.H., Liu, W.T., Bi, Y.L., Du, L., Zhang, C., et al. 2015. Multiple resistance to ACCase and AHAS-inhibiting herbicides in shortawn foxtail (*Alopecurus aequalis* Sobol.) from China. *Pesticide Biochemistry and Physiology* 124:66-72.
- Han, X.J., Dong, Y., Sun, X.N., Li, X.F., and Zheng, M.Q. 2012b. Molecular basis of resistance to tribenuron-methyl in *Descurainia Sophia* (L.) populations from China. *Pesticide Biochemistry and Physiology* 104:77-81.
- Han, H., Yu, Q., Purba, E., Li, M., Walsh, M., Friesen, S., et al. 2012a. A novel amino acid substitution Ala-122-Tyr in ALS confers high-level and broad resistance across ALS-inhibiting herbicides. *Pest Management Science* 68:1164-1170.

- Heap, I. 2021. The international survey of herbicide resistant weeds. Available at <http://www.weedscience.org> (accessed 16 April 2021).
- Li, Q., Du, L., Yuan, G.H., Guo, W.L., Li, W., and Wang, J.X. 2016. Density effect and economic threshold of Japanese brome (*Bromus japonicus* Houtt.) in wheat. *Chilean Journal of Agricultural Research* 76:441-447.
- Li, J., Gao, X.X., Li, M., and Fang, F. 2019. Resistance evolution and mechanisms to ALS-inhibiting herbicides in *Capsella bursa-pastoris* populations from China. *Pesticide Biochemistry and Physiology* 159:17-21.
- Li, Q., Tan, J.N., Li, W., Yuan, G.H., Du, L., Ma, S., et al. 2015. Effects of environmental factors on seed germination and emergence of Japanese brome (*Bromus japonicus*). *Weed Science* 63:41-646.
- Liu, W.T., Bi, Y.L., Li, L.X., Yuan, G.H., and Wang, J.X. 2013. Molecular basis of resistance to tribenuron in water starwort (*Myosoton aquaticum*) populations from China. *Weed Science* 61:390-395.
- Liu, W.T., Yuan, G.H., Du, L., Guo, W.L., Li, L.X., Bi, Y.L., et al. 2015. A novel Pro197Glu substitution in acetolactate synthase (ALS) confers broad-spectrum resistance across ALS inhibitors. *Pesticide Biochemistry and Physiology* 117:31-38.
- Neve, P., Busi, R., Renton, M., and Vila-Aiub, M.M. 2014. Expanding the eco-evolutionary context of herbicide resistance research. *Pest Management Science* 70:1385-1393.
- Ntoanidou, S., Madesis, P., Diamantidis, G., and Eleftherohorinos, I. 2017. Trp574 substitution in the acetolactate synthase of *Sinapis arvensis* confers cross-resistance to tribenuron and imazamox. *Pesticide Biochemistry and Physiology* 142:9-14.
- Ntoanidou, S., Madesis, P., and Eleftherohorinos, I. 2019. Resistance of *Rapistrum rugosum* to tribenuron and imazamox due to Trp574 or Pro197 substitution in the acetolactate synthase. *Pesticide Biochemistry and Physiology* 154:1-6.
- Powles, S.B., and Yu, Q. 2010. Evolution in action: plants resistant to herbicides. *Annual Review of Plant Biology* 61:317-347.
- Tranel, P.J., Wright, T.R., and Heap, I. 2022. Mutations in herbicide-resistant weeds to ALS inhibitors. Available at <http://www.weedscience.org/Mutations/MutationDisplayAll.aspx> (accessed 14 January 2022).
- Vigueira, C.C., Olsen, K.M., and Caicedo, A.L. 2013. The red queen in the corn: agricultural weeds as models of rapid adaptive evolution. *Heredity* 110:303-311.
- Walter, K.L., Strachan, S.D., Ferry, N.M., Albert, H.H., Castle, L.A., and Sebastian, S.A. 2014. Molecular and phenotypic characterization of *Als1* and *Als2* mutations conferring tolerance to acetolactate synthase herbicides in soybean. *Pest Management Science* 70:1831-1839.
- Wang, Q., Ge, L., Zhao, N., Zhang, L.L., You, L.D., Wang, D.D., et al. 2019. A Trp-574-Leu mutation in the acetolactate synthase (ALS) gene of *Lithospermum arvense* L. confers broad-spectrum resistance to ALS inhibitors. *Pesticide Biochemistry and Physiology* 158:12-17.
- Xu, Y.F., Xu, L.N., Li, X.F., and Zheng, M.Q. 2020. Investigation of resistant level to tribenuron-methyl, diversity and regional difference of the resistant mutations on acetolactate synthase (ALS) isozymes in *Descurainia sophia* L. from China. *Pesticide Biochemistry and Physiology* 169:104653.
- Yu, Q., Han, H., and Powles, S.B. 2008. Mutations of the ALS gene endowing resistance to ALS-inhibiting herbicides in *Lolium rigidum* populations. *Pest Management Science* 64:1229-1236.
- Yu, Q., Han, H., Vila-Aiub, M.M., and Powles, S.B. 2010. AHAS herbicide resistance endowing mutations: effect on AHAS functionality and plant growth. *Journal of Experimental Botany* 61:3925-3934.
- Yu, Q., and Powles, S.B. 2014. Resistance to AHAS inhibitor herbicides: current understanding. *Pest Management Science* 70:1340-1350.
- Yuan, G., Tian, Z., Li, T., Qian, Z., Guo, W., and Shen, G. 2021. Cross-resistance pattern to ACCase-inhibiting herbicides in a rare Trp-2027-Ser mutation Chinese sprangletop (*Leptochloa chinensis*) population. *Chilean Journal of Agricultural Research* 81:62-69.
- Zhao, B., Fu, D., Yu, Y., Huang, C., Yan, K., Li, P., et al. 2017a. Non-target-site resistance to ALS-inhibiting herbicides in a *Sagittaria trifolia* L. population. *Pesticide Biochemistry and Physiology* 140:79-84.
- Zhao, Z.Y., Guo, W.L., Li, R.R., Li, Q., Zhao, N., and Wang, J.X. 2017b. Biological activities of herbicides to Japanese brome grass *Bromus japonicus* in wheat fields. *Journal of Plant Protection* 44:841-848.
- Zhao, N., Yan, Y., Wang, H., Bai, S., Wang, Q., Liu, W., et al. 2018. Acetolactate synthase overexpression in mesosulfuron-methyl-resistant shortawn foxtail (*Alopecurus aequalis* Sobol.): Reference gene selection and herbicide target gene expression analysis. *Journal of Agricultural and Food Chemistry* 66:9624-9634.
- Zheng, D.M., Kruger, G.R., Singh, S., Davis, V.M., Tranel, P.J., Weller, S.C., et al. 2011. Cross-resistance of horseweed (*Conyza canadensis*) populations with three different ALS mutations. *Pest Management Science* 67:1486-1492.