

Research Article

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Comparative analysis of resistance to ALS-inhibiting herbicides in smallflower umbrella sedge (*Cyperus difformis*) populations from direct-seeded and puddled-transplanted rice systems

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Abstract

The transition from puddled-transplanted rice (*Oryza sativa* L.) (PTR) to direct-seeded rice (DSR) is gaining popularity in central China. In contrast, the PTR system is the most common practice in southwest China. Weeds are a major problem in the paddy fields of the DSR systems, and herbicides are widely used for weed control. However, the increased frequency and rate of herbicide use leads to the rapid evolution of resistance. Smallflower umbrella sedge (*Cyperus difformis* L.) is a troublesome weed species in rice fields of China and is usually controlled by the acetolactate synthase (ALS)-inhibiting herbicide bensulfuron-methyl. Here, we collected 32 *C. difformis* populations from DSR systems (Hunan Province) and PTR systems (Guangxi Province) and investigated their resistance to bensulfuron-methyl. Results revealed 80% (8 out of 10) populations from Hunan Province and 14% (3 out of 22) populations from Guangxi Province had evolved resistance to bensulfuron-methyl. Five populations from Hunan Province (HN-2, HN-3, HN-5, HN-9, HN-10) possessing the Trp-574-Leu mutation had high-level resistance (ranging from 169- to >1,309-fold) based on GR₅₀ ratios. The resistant populations from Guangxi Province had a lower level of resistance to bensulfuron-methyl due to a Pro-197-Ser mutation. The Asp-376-Glu mutation was only identified in the HN-4 population. In addition, the GX-3 population from the PTR systems was resistant to bensulfuron-methyl without ALS gene mutations, indicating non-target site resistance (NTSR). Although some resistant populations of both regions exhibited cross-resistance to multiple ALS-inhibiting herbicides, including pyrazosulfuron-ethyl, bispyribac-sodium, penoxsulam, and imazapic, sensitivity was also detected to the auxin herbicide MCPA and the photosystem II-inhibiting herbicides bentazone and propanil. These results indicate that cultivation practices affect resistance evolution in *C. difformis*. DSR systems exert high selection pressure by selecting the Trp-574-Leu mutation, resulting in high-level resistance. In contrast, a mutation at Pro-197 plus NTSR likely plays a significant role in ALS resistance in the PTR systems.

Introduction

Rice (*Oryza sativa* L.) is one of the most important food crops in the world. Globally, China is the leading rice producer and consumer (Tian et al. 2018). The direct-seeded rice (DSR) and puddled-transplanted rice (PTR) systems are two different rice cultivation systems used in China. The DSR system is gradually replacing the traditional PTR system due to labor shortages and water scarcity (Tao et al. 2016; Wang et al. 2017). In the PTR system, transplanted rice seedlings have a competitive advantage over weeds, because flooding inhibits the early germination of a large number of weeds after rice seedlings are transplanted (Phukan et al. 2021; Chhun et al. 2019). Furthermore, standing water in the PTR system can suppress the germination of many weed species. In contrast, in the DSR system, weeds emerge simultaneously with rice and grow more rapidly than rice seedlings, resulting in competition for nutrients, water, and light (Kumar and Ladha 2011). Therefore, it is easier for weeds to invade the DSR system than

the PTR system, which can result in up to 100% rice yield loss in DSR (Chauhan 2012; Rao et al. 2007; Singh et al. 2014).

Smallflower umbrella sedge (*Cyperus difformis* L.), a highly self-pollinating C₃-type annual-emerging aquatic species, is widely distributed in both tropical and warm temperate regions of 47 countries (Holm et al. 1991). Due to rice cropping-pattern changes, *C. difformis* has become one of the main troublesome sedge weeds infecting the DSR system areas along the lower-middle Yangtze River, China (Rao et al. 2007). The biological characteristics of *C. difformis* include strong tillering capacity, high fecundity, and growth conditions similar to those of the DSR system, resulting in heavy infestations in DSR system fields compared with PTR system fields (Chauhan and Johnson 2009).

Cyperus difformis is mainly controlled by acetolactate synthase (ALS)-inhibiting herbicides such as bensulfuron-methyl, pyrazosulfuron, penoxsulam, and bispyribac-sodium. Other herbicides (e.g., bentazone, MCPA, and propanil) are also used for control. With increasing labor costs for hand weeding, the increase in herbicide use is expected to continue (Huang et al. 2017). Consequently, due to overreliance on herbicides, many weed species in rice fields have evolved resistance to common herbicides such as penoxsulam, quinclorac, cyhalofop-butyl, bensulfuron-methyl, and propanil (Liu et al. 2019b; Pedroso et al. 2016; Peng et al. 2019; Wei et al. 2019; Yang et al. 2021; Zhao et al. 2017). In particular, *C. difformis* populations have evolved resistance to ALS-inhibiting herbicides such as bensulfuron-methyl, pyrazosulfuron-ethyl, halosulfuron, penoxsulam, and bispyribac-sodium (Kuk et al. 2004; Li et al. 2020; Loddo et al. 2018; Tehranchian et al. 2015b).

Resistance to ALS-inhibiting herbicides can be endowed by either ALS target-gene mutations or non-target enhanced herbicide metabolism, which correspond to target-site resistance (TSR) and non-target site resistance (NTSR) (Powles and Yu 2010). ALS gene mutations have been commonly reported for ALS-inhibitor resistance (Tranel and Wright 2002). Previous reports have demonstrated amino acid substitutions at eight conserved positions of the ALS gene (Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654) that confer resistance to ALS-inhibiting herbicides (Murphy and Tranel 2019). Mutations at Pro-197 and Trp-574 have been reported in *Cyperus* species with resistance to ALS-inhibiting herbicides (Ntoanidou et al. 2016; Riar et al. 2015; Tehranchian et al. 2015a, 2015b; Yamato et al. 2013). Amino acid substitutions by His, Ser, and Ala at position Pro-197 have been reported to confer resistance to ALS-inhibiting herbicides in *C. difformis* populations (Ntoanidou et al. 2016; Tehranchian et al. 2015b). Recently, Pro-197-Ser/Arg/Leu, Asp-376-Glu, and Trp-574-Leu mutations have been reported in resistant *C. difformis* populations from China (Li et al. 2020).

NTSR due to enhanced metabolic resistance to ALS inhibitors occurs widely in weed species (Han et al. 2016). Resistance mechanisms other than TSR also provide metabolic resistance, which may result in resistance to multiple herbicide modes of action (Laforest et al. 2021). Metabolic resistance is the most important NTSR, and its related functional genes include cytochrome P450 monooxygenases (P450s), glycosyltransferases, glutathione S-transferases, and ATP-binding cassette transporters (Délye 2013; Yu and Powles 2014; Yuan et al. 2007). P450s play an important metabolic role in plants under herbicide stress (Bak et al. 2011). In most cases of ALS-inhibiting herbicide resistance, P450-mediated herbicide metabolism has been verified in various plant species such as wheat (*Triticum aestivum* L.), rice, blackgrass

(*Alopecurus myosuroides* Huds.), and rigid ryegrass (*Lolium rigidum* Gaudin) (Dimaano and Iwakami 2021; Franco-Ortega et al. 2021; Han et al. 2021). In recent years, many reports also demonstrated that TSR and P450-mediated metabolic resistance coexist in weed species resistant to ALS-inhibiting herbicides (Iwakami et al. 2014; Yang et al. 2018; Yu et al. 2009). The involvement of P450s in herbicide metabolism has been confirmed by indirect evidence (Busi et al. 2017). For example, the P450 inhibitor malathion was used to detect herbicide metabolism indirectly in resistant populations (Guo et al. 2015; Kaspar et al. 2011; Zhao et al. 2019).

Bensulfuron-methyl, an ALS-inhibiting herbicide, is widely used to control *C. difformis* in the rice fields of China. In recent years, farmers have complained that field applications of bensulfuron-methyl have been ineffective in controlling *C. difformis* populations in Hunan Province, well known to have the most DSR production system areas. However, the PTR system is the primary planting system used in Guangxi Province, China. Therefore, *C. difformis* seeds were harvested from major rice-producing regions of Hunan and Guangxi provinces, China. Following seed harvesting, studies were implemented to compare and investigate [1] *C. difformis* resistance levels to the ALS-inhibiting herbicide bensulfuron-methyl, [2] cross-resistance to pyrazosulfuron, bispyribac-sodium, penoxsulam, and imazapic, and [3] multiple resistance to bentazone, propanil, and MCPA to understand the potential mechanisms of resistance to ALS-inhibiting herbicides.

Materials and Methods

Plant Material

Cyperus difformis seeds were collected from rice fields of Hunan and Guangxi provinces, where ALS-inhibiting herbicides have been used for at least 15 yr. Susceptible (S) populations (HN-S and GX-S) were harvested from crop regions without herbicide exposure. The sensitivity assays of S populations were performed at half the recommended dose of bensulfuron-methyl in the field. The sample information, including geographic distribution, field coordinates, and cultivation method, were recorded (Figure 1; Table 1). The harvested *C. difformis* seeds were air-dried and stored at 4 C until they were used in experiments.

Single-Rate Herbicide Test

The seeds of all populations were germinated in petri plates containing 20 ml of 0.6% agar at room temperature. After emergence, young seedlings were transplanted into 22-cm (length) × 12.5-cm (width) × 7-cm (depth) plastic pots containing loam soil with 40 seedlings per pot. The seedlings were grown in a greenhouse under natural sunlight and were supplemented with instant NPK fertilizer (N ≥ 13.5%, P₂O₅ ≥ 15%, K₂O ≥ 15%) once every 2 wk and watered daily. At the 2- to 3-leaf stage, plants were sprayed with bensulfuron-methyl at 45 g ai ha⁻¹. Herbicide applications were conducted in a moving sprayer equipped with one TPV 9501E flat-fan nozzle (Guangxi Pastoral Biochemistry, Inc., Nanning, Guangxi, China 530000) calibrated to deliver 675 L ha⁻¹ at 0.4 MPa. Plant survival was recorded as a percent at 28 d after treatment (DAT), and the mature seeds of suspected resistant (R) populations were collected to air-dry for screening experiments.

Dose-Response Experiments

Seeds from each population were evenly distributed on the 0.6% agar to germinate (16-h light/8-h dark, 35/25 C, with ~75% relative

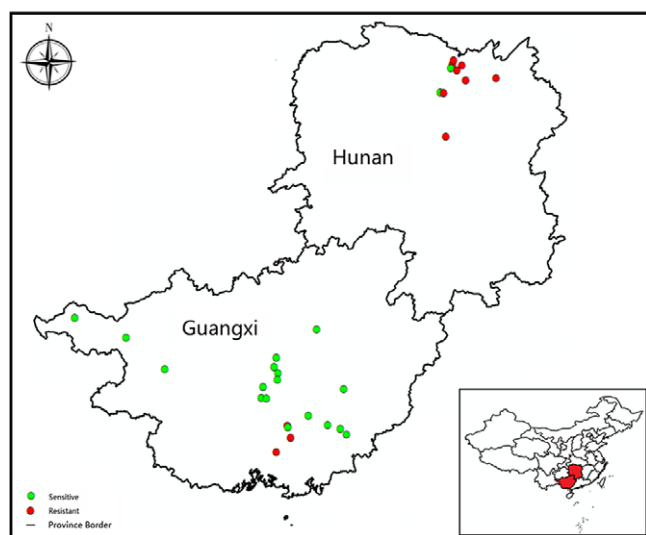


Figure 1. The distribution and resistance profiles to bensulfuron-methyl of *Cyperus difformis* populations in this study.

humidity) for 2 wk and were then transplanted into 10-cm-diameter plastic pots filled with loam soil (15 seeds per pot) and cultured in a greenhouse as previously described. After 1 wk, seedlings were thinned to 10 uniformly sized seedlings per pot (three repetitions per treatment). At the 2- or the 3-leaf stage, *C. difformis* populations were treated with varying rates of bensulfuron-methyl at 0, 0.7, 1.4, 2.8, 5.6, and 11.2 g ai ha⁻¹ for S populations; 0, 22.5, 45.0, 90.0, 180.0, and 360.0 g ai ha⁻¹ for HN-8 from Hunan Province and R populations from Guangxi Province; and 0, 45.0, 90.0, 180.0, 360.0, 720.0, 1,440.0, and 2,880.0 g ai ha⁻¹ for R populations from Hunan Province. The experiment was designed as a randomized complete block with three replications per treatment. At 28 DAT, plant survival was recorded, and the aboveground parts of the plant were harvested and then weighed after being oven-dried for 72 h at 80 C.

Susceptibility to Multiple Herbicides

The susceptibility of bensulfuron-methyl R populations, which were the offspring of survivors from bensulfuron-methyl-treated populations, was tested against multiple herbicides with different modes of action (Table 2). *Cyperus difformis* populations were planted as described earlier. Following germination, 40 seedlings per pot were planted for each R *C. difformis* population. At the 3- to 4-leaf stage, plants were sprayed with the herbicides at recommended doses (Table 2).

ALS Gene Sequencing

Total RNA from leaf tissue was extracted from untreated S and R plants (10 plants per population) using the UNI-Q-10 Column Trizol Total RNA Isolation Kit (Sangon Biotech, Shanghai, China). RNA was quantified using the NanoDrop 2000 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was synthesized from 1 µg of RNA using the First Strand cDNA Synthesis Kit (Sangon Biotech). To identify the potential mutation in the conserved ALS gene regions, five pairs of primers (Table 3) were designed and synthesized based on the ALS sequence of *C. difformis* (GenBank EF061294.2). The primers were able to amplify fragments with theoretical sizes of 596, 357, 488, 946,

Table 1. Recorded *Cyperus difformis* populations information in this study.

Population	Location	Latitude, longitude	Cultivation method ^a
HN-1	Mushan town, Hanshou County	28.83°N, 111.95°E	DSR system
HN-2	Dananhu town, Hanshou County	28.83°N, 112.00°E	DSR system
HN-3	Zhoukou town, Hanshou County	28.02°N, 112.05°E	DSR system
HN-4	Guantouzui town, Hanshou County	29.10°N, 112.97°E	DSR system
HN-5	Ankang town, Anxiang County	29.35°N, 112.17°E	DSR system
HN-6	Xiayukou town, Anxiang County	29.29°N, 112.14°E	DSR system
HN-7	Shenliuguan town, Anxiang County	29.42°N, 112.19°E	DSR system
HN-8	Nanzhou town, Nan County	29.33°N, 112.35°E	DSR system
HN-9	Wushenggong town, Nan County	29.24°N, 112.25°E	DSR system
HN-10	Caowei town, Yuanjiang County	29.06°N, 112.42°E	DSR system
GX-1	Fengling Road, Shanglin County	23.44°N, 108.69°E	PTR system
GX-2	Changlian town, Shanglin County	23.24°N, 108.67°E	PTR system
GX-3	Pinnan town, Lingshan County	22.51°N, 109.20°E	PTR system
GX-4	Luwei town, Lingshan County	22.24°N, 108.94°E	PTR system
GX-5	Binzhou town, Binyang County	23.22°N, 108.76°E	PTR system
GX-6	Silian town, Xincheng County	23.97°N, 108.94°E	PTR system
GX-7	Heli town, Xingbin District	23.69°N, 108.97°E	PTR system
GX-8	Lingnan town, Hesun County	23.81°N, 108.90°E	PTR system
GX-9	Luorong town, Yufeng District	24.49°N, 109.68°E	PTR system
GX-10	Shapu town, Liucheng County	24.55°N, 109.37°E	PTR system
GX-11	Liantang town, Heng County	22.72°N, 109.14°E	PTR system
GX-12	Qianjiang town, Xinning District	23.57°N, 108.95°E	PTR system
GX-13	Daling town, Qintang District	22.91°N, 109.52°E	PTR system
GX-14	Baiyu town, Tianyang County	23.76°N, 106.88°E	PTR system
GX-15	Tiandeng town, Tiandeng County	23.06°N, 107.17°E	PTR system
GX-16	Erhuanbei Road, Yuzhou County	22.66°N, 110.12°E	PTR system
GX-17	Kuiye town, Xingye County	22.73°N, 109.88°E	PTR system
GX-18	Leli town, Tianlin County	24.34°N, 106.18°E	PTR system
GX-19	Xingdaohu town, Hepu County	22.70°N, 109.15°E	PTR system
GX-20	Liangtian town, Luchuan County	22.56°N, 110.22°E	PTR system
GX-21	Tangbu town, Teng County	23.39°N, 110.17°E	PTR system
GX-22	Deer town, Longlin County	24.70°N, 105.27°E	PTR system

^aDSR, direct-seeded rice; PTR, puddled-transplanted rice.

and 1,934 bp, containing seven codons of Ala-122, Pro-197, Ala-205, Asp-376, Trp-574, Pro-653, and Ser-654 (Tehranchian et al. 2015b). Four cDNA sequences amplified using four primer

Table 2. Herbicides and applied rates in single-dose experiments.

Group ^a	Herbicides	Site of action ^b	Formulation ^c	Manufacturer	Recommended field dose —g ai ha ⁻¹ —
SU	Bensulfuron-methyl	ALS	30% WP	DuPont, Shanghai, China	45
	Pyrazosulfuron	ALS	10% WP	Jiangsu Hormone Research Institution, Jiangsu, China	30
TP	Penoxsulam	ALS	25 g L ⁻¹ OD	Dow AgroSciences, Beijing, China	17.5
PTB	Bispyribac-sodium	ALS	100 g L ⁻¹ SC	Jiangsu Futian Agrochemical, Jiangsu, China	30
IMI	Imazapic	ALS	240 g L ⁻¹ SL	BASF, Shanghai, China	72
Benzothiadiazinone	Bentazone	PSII-His-215	480 g L ⁻¹ SL	BASF, Jiangsu, China	1440
Auxin	MCPA	Auxin mimics	750 g L ⁻¹ AS	Nufarm, Shanghai, China	562.5
Amides	Propanil	PSII-Ser-264	34% EC	Xianda, Shandong, China	4080

^aIMI, imidazolinone; PTB, pyrimidinyl-thiobenzoate; SU, sulfonyleurea; TP, triazolopyrimidine.

^bALS, acetolactate synthase; PSII, photosystem II.

^cAS, aqueous solutions; OD, oil dispersion; SC, suspension concentrate; SL, soluble concentrate; WP, wettable powder.

Table 3. Primer pairs designed for amplification of *Cyperus difformis* ALS gene.

Number	Primer	Sequence (5'→3')	T	Amplicon size	Targeted mutations
1	ALSf2	ATGTTCTCGTTGAGGTTCTC	—C— 53	— bp— 596	Ala-122, Pro-197, Ala-205
	ALSR597	TGATTCCAGAAACAAGACGGA			
2	ALSf75	ATTCACCAAGCCCTTACGAG	55	357	Pro-197, Ala-205
	ALSR431	GAAGTGGCCAAGAAAAATGC			
3	ALSf546	GCAACCTGAAGACAACCAGC	57	488	Asp-376
	ALSR1033	CCAGTTCAGCCCTCCAACCTA			
4	ALSf729	ACTGTGTCTGCGCTTGTG	56	946	Asp-376, Trp-574
	ALSR1674	CTTGAATGCCCTCCACTTG			
5	allALSf	ATCCAAGCACTCAAACCTCCT	56	1934	Ala-122, Pro-197, Ala-205, Asp-376, Trp-574, Pro-653, Ser-654
	allALSR	AGCCTACCATCAGAAAGTCAA			

pairs (1, 2, 3, and 4 in Table 3) could be assembled into a 1,673-bp sequence. In addition, the 5th primer pair (allALSf and allALSR) was used to amplify a full-length sequence containing seven known mutation sites.

PCR amplification was conducted in a 25- μ l volume containing 1 μ l of cDNA (100 ng μ l⁻¹), 1 μ l of each primer (10 μ M), 9.5 μ l of double-distilled water (ddH₂O), and 12.5 μ l of 2 \times Taq PCR StarMix with Loading Dye (GenStar, Beijing, China). PCR was performed on the thermal cycling system (Applied Biosystems, Foster City, CA, USA) with the following profile: denaturing at 94 C for 4 min; then 40 cycles of 94 C for 30 s, annealing for 30 s at 53, 55, 57, 56, and 56 C for the five primer pairs, respectively (Table 3), and 72 C for 60 s; followed by a final extension step of 10 min at 72 C. Amplified PCR products were sequenced by Sangon Biotech. The sequencing data were visually checked and aligned using multiple sequence comparisons in DNAMAN v. 7.1 software (Edgar 2004).

Metabolic Resistance Screen

The enhanced metabolism of P450s is one of the reasons for metabolic resistance in resistant populations. Malathion (P450 inhibitor) was used to test metabolic resistance to bensulfuron-methyl in R populations of *C. difformis*. According to our preliminary experiments, 1,000 g ai ha⁻¹ of malathion was applied 2 h before bensulfuron-methyl treatment. *Cyperus difformis* was cultivated following the same method described earlier. Briefly, 40 seedlings of R offspring (2- to 3-leaf stage) were sprayed with malathion plus bensulfuron-methyl or with bensulfuron-methyl alone in a

greenhouse. The rate of surviving plants was recorded as a percentage at 28 DAT.

Statistical Analysis

Data obtained from the dose-response experiments were subjected to a nonlinear regression analysis using a three-parameter equation (Equation 1) in Sigma Plot v. 12.5 (Systat Software, San Jose, CA, USA). The GR₅₀ value (the herbicide dose resulting in 50% growth inhibition) was calculated as follows:

$$y = a / \left[1 + \left(\frac{x}{x_0} \right)^b \right] \quad [1]$$

where x is the herbicide concentration, y is the dry biomass percentage of the untreated control, x_0 is GR₅₀, a is a constant, and b is the slope of the curve.

The resistance index (RI) was calculated as the GR₅₀ of the R population divided by the GR₅₀ of the S population.

In the metabolic resistance experiments, data for bensulfuron-methyl metabolism were analyzed by independent-samples t -test ($P < 0.05$) in SPSS v. 22.0 (IBM, Armonk, NY, USA) to determine whether the use of malathion would affect the populations resistant to herbicides.

Table 4. Parameter values of dose–response curves to bensulfuron-methyl for *Cyperus difformis*.

Population	Regression parameters			GR ₅₀	Resistance index
	<i>a</i>	<i>b</i>	R ²		
HN-2	104 (5.3)	0.7(0.09)	0.90	372 (80.8)	169
HN-3	—	—	—	>2,880	>1,309
HN-4	99.7(0.2)	1.2 (0.01)	0.99	209 (3.1)	94.9
HN-5	104 (0.3)	0.7(0.01)	0.97	1,071 (27.3)	487
HN-7	101(4.4)	0.9 (0.1)	0.95	111(16.8)	50.3
HN-8	94.8 (5.5)	2.7(0.6)	0.93	69.8(7.4)	31.7
HN-9	—	—	—	>2,880	>1,309
HN-10	—	—	—	>2,880	>1,309
GX-3	101 (3.5)	2.6 (0.3)	0.97	68.7(4.0)	31.2
GX-4	100 (4.8)	1.6 (0.3)	0.94	39.0 (5.9)	17.7
GX-11	99.5 (5.2)	1.7 (0.3)	0.95	28.0 (3.3)	12.7
HN-S	95.2 (4.0)	2.5 (0.4)	0.97	2.2 (0.2)	—
GX-S	96.4 (2.8)	2.3 (0.3)	0.98	3.5 (0.2)	—

Results and Discussion

Herbicide Response Experiments of Bensulfuron-Methyl

A total of 32 *C. difformis* populations were tested with bensulfuron-methyl at the recommended dose (45 g ai ha⁻¹). The two S populations, HN-S and GX-S, died at this herbicide rate, while populations that survived this rate were considered resistant. In this study, 11 of the 32 populations survived this rate with variable survivorship ranging from 10% to 100% (data not shown), suggesting that these populations had evolved different resistance levels to bensulfuron-methyl. Of the 10 populations from the DSR system of Hunan Province, 8 were resistant to bensulfuron-methyl, and 3 out of those 8 R populations (HN-3, HN-9, and HN-10) had 100% survival at the recommended dose. There were only three R populations from the PTR systems of Guangxi Province with a survival rate of <30%. Based on these findings, 2 S populations (HN-S and GX-S) and 11 putative R populations (HN-2, HN-3, HN-4, HN-5, HN-7, HN-8, HN-9, HN-10, GX-3, GX-4, and GX-11) were studied further in the following experiments.

Compared with the S populations, the putative R populations were confirmed and exhibited various resistance levels to bensulfuron-methyl (Table 4). The GR₅₀ value for R biotypes ranged from 28.0 to >2,880 g ai ha⁻¹ (Table 4), while the two S biotypes showed a similar GR₅₀ value lower than the recommended dose. The RI of Hunan Province populations for bensulfuron-methyl ranged from 31.7- to >1,309-fold. Meanwhile, the RI of Guangxi Province populations showed from 12.7- to 31.2-fold resistance to bensulfuron-methyl compared with the S populations. The highest and lowest resistance levels to bensulfuron-methyl were exhibited by HN-9 and GX-11, respectively (Figure 2). These results reveal that the DSR system's R populations from Hunan Province show higher resistance levels than those from the Guangxi Province PTR system.

This is the first study to compare herbicide-resistance evolution in *C. difformis* from DSR and PTR systems in China. The frequency and level of resistance to bensulfuron-methyl were much higher in *C. difformis* populations in the DSR system fields of Hunan Province than in the PTR system fields of Guangxi Province. Several factors potentially explain this situation. The transition from the PTR to DSR system in Hunan Province has resulted in overreliance on herbicides. ALS-inhibiting herbicides are applied up to four times annually in the rice fields of Hunan Province

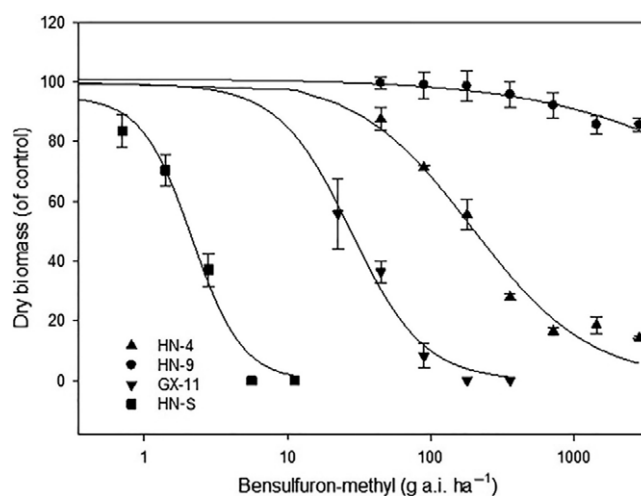


Figure 2. Dose–response curves for bensulfuron-methyl–susceptible (HN-S) and bensulfuron-methyl–resistant (HN-4, HN-9, GX-11) *Cyperus difformis* populations based on aboveground dry biomass 28 d after treatment.

(Liu et al. 2019b). In contrast, the PTR system is adopted in most areas of Guangxi Province, where the fields are continuously flooded throughout the growing season (Ling et al. 2021). The water layer easily controls *C. difformis* by inhibiting the germination of weed seeds. In addition, paddy fields in Guangxi Province are small and scattered, making it easier to manage weeds with traditional manual labor (Wu et al. 2020).

The herbicide bensulfuron-methyl had been used for weed control in Hunan Province since the 1990s, much earlier than in Guangxi Province (Li and Chen 1991; Wang et al. 1999). The repetitive and continuous use of ALS-inhibiting herbicides for the last 15 yr led to the development of herbicide resistance in weeds of the rice fields in China (Liu et al. 2019b; Wu et al. 2005). In recent years, the resistance of weeds to ALS-inhibiting herbicides has also been reported in a few paddy fields of Hunan Province. (Li et al. 2020; Liu et al. 2019a; Peng et al. 2019). In fact, resistant *C. difformis* populations have been one of the most troublesome weed problems in Hunan Province. These results indicate that resistance evolution in *C. difformis* is related to both cultural methods and the repeated use of ALS-inhibiting herbicides.

Table 5. Percentage of surviving plants from resistant (R) and susceptible (S) *Cyperus difformis* populations in response to multiple ALS-inhibiting herbicides.^a

Population	Survival rate			
	Pyrazosulfuron	Penoxsulam	Bispyribac-sodium	Imazapic
	%			
HN-2	35.0	15.0	35.0	40.0
HN-3	75.0	45.0	50.0	65.0
HN-4	65.0	47.5	65.0	75.0
HN-5	77.5	50.0	72.5	60.0
HN-7	50.0	10.0	30.0	10.0
HN-8	12.5	0.0	0.0	12.5
HN-9	100.0	80.0	60.0	82.5
HN-10	85.0	77.5	45.0	65.0
GX-3	77.5	5.0	0.0	7.5
GX-4	45.0	7.5	0.0	5.0
GX-11	30.0	0.0	0.0	2.5
HN-S	0.0	0.0	0.0	0.0
GX-S	0.0	0.0	0.0	0.0

^aForty plants of each population were treated with each herbicide at the field-recommended dose.

Susceptibility to Multiple Herbicides

In this study, the field-recommended dose of multiple ALS-inhibiting herbicides was used to test the cross-resistance in R populations. The HN-8 and GX-11 populations showed cross-resistance to sulfonylurea (SU, pyrazosulfuron) and imidazolinone (IMI, imazapic), while GX-3 and GX-4 had cross-resistance to SU (pyrazosulfuron), triazolopyrimidine (TP, penoxsulam), and IMI (imazapic). Other R populations exhibited cross-resistance to all herbicides tested (Table 5). Our experiments demonstrated that *C. difformis* populations in China had different resistance levels to bensulfuron-methyl and cross-resistance to multiple ALS inhibitors. Similar results were also reported in *C. difformis* and the sedge species bog bulrush [*Schoenoplectus mucronatus* (L.) Palla], annual sedge (*Cyperus compressus* L.), and rice flatsedge (*Cyperus iria* L.) with different target-site mutations (Li et al. 2020; McCullough et al. 2016; Riar et al. 2015; Yamato et al. 2013).

Additionally, all populations died when field-recommended doses of other herbicides with non-ALS inhibiting chemistries, such as MCPA, bentazone, and propanil, were applied. This indicates that these populations were still susceptible to MCPA, bentazone, and propanil. Based on these results, we suggest that farmers could also use a mixture of herbicides to control ALS resistance in sedges. For example, the mixture of MCPA and bentazone is an effective way to prevent resistance in *C. difformis* via foliar application. Previously, herbicide mixtures have been reported as one of the most effective methods for management of resistant weeds (Comont et al. 2020).

Sequencing and Analysis of ALS Gene

Ten individual plants per population were amplified using five pairs of primers, and the sequencing results were compared with the full-length ALS gene of R and S populations. PCR products containing base mutation sequences were purified and transformed into *Escherichia coli* to select several monoclonal clones for cloning and sequencing to confirm the correctness of the mutation sites. The sequence with point mutations detected by cloning and sequencing was confirmed to endow resistance to ALS-inhibiting herbicides. All assembled sequences from different populations had approximately 99.5% similarity to the

previously published ALS gene of *C. difformis* (GenBank accession EF061294.2).

The alignment of the ALS cDNA sequence showed Pro-197-Leu/Ser mutations in five populations (HN-2, HN-4, HN-8, GX-4, and GX-11), an Asp-376-Glu mutation in three populations (HN-4, HN-7, and HN-10), and a Trp-574-Leu mutation in five populations (HN-2, HN-3, HN-5, HN-9, and HN-10). The Trp-574-Leu mutation was found in more than 70% of individuals in the three populations with higher resistance levels (HN-3, HN-9, and HN-10). That means the frequency of Trp-574-Leu mutations was the highest in the DSR systems from Hunan Province, but this mutation was not identified in the PTR system from Guangxi Province. Pro-197-Ser mutations were identified in two populations (GX-4 and GX-11) from PTR systems. In addition, none of the populations contained any mutation at Pro-653 and Ser-654, and no ALS gene mutation was detected in the R population of GX-3.

Generally, TSR depends on precise mutations, the ALS-inhibiting herbicide's structure, and the weed species. It has been reported that eight ALS gene mutations conferring resistance to ALS-inhibiting herbicides have been identified in resistant plants (Murphy and Tranel 2019). The target-site mutation at codon Pro-197 could have substitutions of Ala, Ser, Arg, and His, which confer cross-resistance to different ALS inhibitors in *C. difformis* (Li et al. 2020). In this study, the populations with Pro-197-Ser were cross-resistant to penoxsulam, imazapic, and bispyribac-sodium. As previously documented, the Pro-197-Ser substitution results in broad-spectrum resistance to SU, TP, PTB (pyrimidinyl-thiobenzoate), or SCT (sulfonyl-aminocarbonyl-triazolinone) herbicides but low or no resistance to IMI herbicides in weeds (Zhao et al. 2017). Pro-197-Ser/Leu/Arg substitution in *C. difformis* from Anhui Province in China also conferred cross-resistance to penoxsulam, but plants were sensitive to imazapic and bispyribac-sodium (Li et al. 2020).

The Asp-376-Glu mutation was also reported in three R *C. difformis* populations from China. In this work, five out of eight R populations from Hunan Province were identified as Trp-574-Leu mutants, which revealed the highest resistance level and broad-spectrum resistance to all five classes of ALS-inhibiting herbicides (Table 6). A recent study reported that the Trp-574-Leu mutation was detected in 2 of the 12 resistant populations collected in 2012 to 2015 from Hunan Province (Li et al. 2020). These results indicate that resistance of *C. difformis* populations has evolved in recent years. Thus, resistance evolution to ALS-inhibiting herbicides needs to be focused on in the long term.

Metabolic Resistance to Bensulfuron-Methyl

When malathion was applied alone at 1,000 g ai ha⁻¹, no significant influence was observed in the growth of either the R or S populations. As described in Table 7, using malathion 2 h before applying bensulfuron-methyl enhanced the efficacy of bensulfuron-methyl in Hunan (HN-2, HN-3, HN-4, and HN-8) and Guangxi populations (GX-3, GX-4, and GX-11). Moreover, the mortality of these populations showed a significant difference after being treated with malathion ($P < 0.05$). This finding indicated that P450s likely contribute to bensulfuron-methyl resistance in *C. difformis*. Other than that, the resistance observed in the HN-5, HN-9, and HN-10 populations was not reversed by malathion. Combined with the changes in mortality, we deduced that the high level of resistance caused by the Trp-574-Leu mutation masked the effect of metabolic resistance. However, these results require further tests

Table 6. Frequency of different codons and derived amino acids in the ALS gene in different *Cyperus difformis* populations.

Populations	Amino acid site			Numbers of plants with specific genotype/total plants identified
	197	376	574	
HN-S	CCT (Pro)	GAT (Asp)	TGG (Trp)	10/10
GX-S	CCT (Pro)	GAT (Asp)	TGG (Trp)	10/10
HN-2	CCT (Pro)	GAT (Asp)	TGG (Trp)	4/10
	CTT (Leu)	—	—	4/10
HN-3	—	—	TTG (Leu)	2/10
	CCT (Pro)	GAT (Asp)	TGG (Trp)	3/10
	—	—	TTG (Leu)	7/10
HN-4	CCT (Pro)	GAT (Asp)	TGG (Trp)	3/10
	TCT (Ser)	—	—	3/10
HN-5	—	GAA (Glu)	—	4/10
	CCT (Pro)	GAT (Asp)	TGG (Trp)	4/10
HN-7	—	—	TTG (Leu)	6/10
	CCT (Pro)	GAT (Asp)	TGG (Trp)	9/10
HN-8	—	GAA (Leu)	—	1/10
	CTT (Leu)	—	—	10/10
HN-9	—	—	TTG (Leu)	10/10
HN-10	CCT (Pro)	GAT (Asp)	TGG (Trp)	1/10
	—	GAA (Leu)	—	1/10
GX-3	—	—	TTG (Leu)	8/10
	CCT (Pro)	GAT (Asp)	TGG (Trp)	10/10
GX-4	CCT (Pro)	GAT (Asp)	TGG (Trp)	1/10
	TCT (Ser)	—	—	9/10
GX-11	CCT (Pro)	GAT (Asp)	TGG (Trp)	2/10
	TCT (Ser)	—	—	8/10

Table 7. Mortality rate and significance test of *Cyperus difformis* populations treated by bensulfuron-methyl in the presence and absence of malathion.

Population	Mortality ^a		Significance
	Bensulfuron-methyl	Bensulfuron-methyl+malathion	
	%		
HN-2	43	72	0.005*
HN-3	33	38	0.038*
HN-4	59	72	0.046*
HN-5	27	16	0.056
HN-7	100	100	—
HN-8	89	100	0.041*
HN-9	5	3	0.070
HN-10	6	7	0.158
GX-3	81	100	0.019*
GX-4	76	84	0.042*
GX-11	94	100	0.045*
HN-S	100	100	—
GX-S	100	100	—

^aData are mean.

*Significant difference at P < 0.05 level by t-test.

to determine whether metabolic resistance works in combination with other NTSR to form herbicide resistance in *C. difformis*.

The GX-3 population with no mutations was resistant to bensulfuron-methyl for several reasons. First, recent research has demonstrated NTSR (especially P450s) can play an essential role in weed resistance to ALS-inhibiting herbicides (Dimaano and Iwakami 2021). Our preliminary experiments showed an apparent synergistic effect of the combined application of bensulfuron-methyl plus malathion on R *C. difformis* populations, which indicated the likely involvement of P450s with resistance in some R populations. Several studies have also demonstrated that P450s were correlated with herbicide resistance in different weed species (Dimaano and Iwakami 2021; Han et al. 2021; Iwakami et al. 2019).

Second, as some members of *Cyperaceae* are polyploid, resistance alleles may be homozygous in the genome (Tejavathi and Nijalingappa 1990). The ploidy and NTSR of *C. difformis* need further studies to evaluate their relationship with resistance in the future.

Plants metabolize herbicides before they reach the active site, which means that the amount of herbicide metabolism is critical for weed control (Khaledi et al. 2019). P450s are key metabolic enzymes present in many crops and weeds and perform metabolic functions (Dimaano and Iwakami 2021; Nandula et al. 2019). Many RNA-seq studies have directly demonstrated that P450s are involved in herbicide metabolism, such as in American slough-grass [*Beckmannia syzigachne* (Steud.) Fernald], flixweed [*Descurainia sophia* (L.) Webb ex Prantl], and *Echinochloa glabrescens* Munro ex Hook. f. (Pan et al. 2016; Yan et al. 2019; Yang et al. 2018). Therefore, enhanced P450-mediated herbicide metabolism should be further investigated in resistant weed species.

The experiments provide evidence that TSR and P450-mediated metabolic resistance coexist in most R populations. These results are in accordance with previous reports of different weed species' resistance to ALS-inhibiting herbicides (Bai et al. 2019; Han et al. 2016; Yang et al. 2016). Metabolic resistance to ALS-inhibiting herbicides has resulted in rapid evolution (Beckie and Tardif 2012; Laforest et al. 2021). Metabolic herbicide resistance can potentially endow resistance to many herbicides and poses a threat to herbicide sustainability and thus crop production, calling for major research and management efforts. The integrated weed management approach includes the suppression of weeds through nonchemical methods, such as the use of allelopathic rice cultivars, deep flooding, alternation between water- and dry-seeded systems, and crop rotation (Liu et al. 2021). For example, allelopathic rice inhibited resistant barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] more than susceptible cultivars (Yang et al. 2017). Australian grain growers have developed harvest weed seed control (HWSC) practices to reduce the viability of weed seeds by

preventing weed seeds from escaping and entering the seedbank (Somerville et al. 2018; Walsh et al. 2018).

This is the first study to compare *C. difformis* resistance to bensulfuron-methyl in different planting systems in China. Our findings revealed that Trp-574-Leu mutations in ALS inhibitor-resistant *C. difformis* are most common in the PTR system of Hunan Province and result in high-level resistance to ALS-inhibiting herbicides. In contrast, Pro-197-Ser and NTSR are the main reason for resistance in the DSR system. The cross-resistance to ALS-inhibiting herbicides was the result of TSR and NTSR. Management practices to control resistant *C. difformis* populations vary in different cultivation systems. Our study suggests that integrated weed management combined with tillage methods and non-chemical methods to suppress weeds will be critical for delaying the development of ALS inhibitor-resistant weeds in China.

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