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RESEARCH ARTICLE

ACCase and ALS susceptibility status of *Echinochloa* spp. populations in Anhui Province, China, and their baseline sensitivity to the HPPD inhibitor tembotrione

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ABSTRACT

Echinochloa spp. are considered highly detrimental weeds, representing a substantial threat to rice (*Oryza sativa* L.) and maize (*Zea mays* L.) production in Anhui Province, China. Tembotrione, a 4-hydroxyphenylpyruvate dioxygenase-inhibiting herbicide, was newly registered in China in 2021 for managing annual weeds in maize fields. This study aims to assess the susceptibility and extent of resistance in 85 *Echinochloa* spp. populations, collected from diverse rice and maize fields in Anhui Province, to two frequently employed herbicides: The acetyl-CoA carboxylase (ACCase) inhibitor metamifop and the acetolactate synthetase (ALS) inhibitor penoxsulam. The study also seeks to determine their baseline sensitivity to tembotrione, and evaluate the potential risk of herbicide resistance development in these populations. Single-dose testing confirmed that among the 85 populations, 44 were resistant to metamifop, with resistance index (RI) ranging from 2.07 to 10.80; 63 were resistant to penoxsulam, with RI ranging from 2.06 to 16.04; 30 were resistant to both metamifop and penoxsulam. Target gene sequencing revealed a common Trp-574-Leu mutation in five penoxsulam-resistant populations. Subsequently, whole plant dose-response showed that the tembotrione doses causing 50% growth reduction (GR_{50} values) in the 85 populations ranged from 6.34 to 43.94 g ai ha^{-1}. Meanwhile, the frequency of the GR_{50} values was not normally distributed but distributed as a unimodal curve and extending towards the sensitive end. The baseline sensitivity ($GR_{50}b$) to tembotrione was 17.96 g ai ha^{-1} and the baseline sensitivity index (SI_{50}) was 6.93.

Key words: Baseline sensitivity, dose-response assays, *Echinochloa* species, gene mutation, resistance distribution, tembotrione.

INTRODUCTION

The genus *Echinochloa* comprises approximately 50 species of annual or perennial grasses, with the majority distributed in temperate to tropical regions and considered economically important weeds in agricultural fields (Michael, 1983). These species typically exhibit high tillering capacity and strong adaptability, leading to substantial yield losses in crop cultivation (Chauhan and Johnson, 2010). Although *Echinochloa* spp. are known to extensively infest rice (*Oryza sativa* L.) fields globally, they are also predominantly found in some upland crops, such as maize (*Zea mays* L.) and soybean (*Glycine max* (L.) Merr.), due to the changes in crop rotations (Zhao et al., 2020). Several *Echinochloa* species, including *Echinochloa crusgalli* (L.) P. Beauv. var. mitis, *Echinochloa glabrescens*, *Echinochloa colonum*, *Echinochloa hispidula*, *Echinochloa oryzoides* and *Echinochloa caudata* have been common weed species with long history and are difficult to control (Chen et al., 2019). Effective control of these species is essential for ensuring stable crop productivity in these regions.

In the past two decades, the control of *Echinochloa* spp. in rice and maize fields has heavily relied on various chemical herbicides, including acetyl-CoA carboxylase (ACCase) (e.g., metamifop and cyhalofop-butyl), acetolactate synthase (ALS) (e.g., penoxsulam, triafamone, bispyribac-sodium, pyribenzoxim, nicosulfuron and rimsulfuron), auxin mimics (e.g., quinclorac and florpyrauxifen-benzyl), photosystem II (PSII) (e.g., atrazine), and 4-hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.13.11.27) (e.g., tripyrasulfone, mesotrione and topramezone) (Haq et al., 2022, Wang et al., 2024a). However, their long-term and large-scale applications have imparted intense selection pressure on weed, leading to the rapid development of resistance to herbicides with multiple modes of action (MOAs) within *Echinochloa* populations (Yu et al., 2024). Nowadays, herbicide-resistant *Echinochloa* spp. have become a global issue and are frequently identified in many rice and maize cropping countries, including China (Heap, 2023). To mitigate the spread of herbicide-resistant *Echinochloa* species, alternative herbicides with different MOAs and/or low risk of resistance development are urgently required.

Two mechanisms commonly used to explain herbicide resistance are target-site resistance (TSR) and non-target-site resistance (NTSR). The TSR is generally caused by target-site mutations and over expression of target enzyme genes. According to previous research, at least nine and seven codon sites were identified to be able to mutated in the *ALS* and the *ACCase* genes, respectively, and thus conferring ALS and the ACCase inhibiting herbicides resistance (Fang et al., 2022; Yin et al., 2023). Target-site mutations often endow weeds with a high resistance level and cross-resistance in various weed species, such as Chinese sprangletop (*Leptochloa chinensis* (L.) Nees), American sloughgrass (*Beckmannia syzigachne* (Steud.) Fernald), and *E. crus-galli* (Fang et al., 2022; Jiang et al., 2022; Yin et al., 2023). The NTSR can also endow herbicide-resistance (Wang et al., 2024b). For example, proliferative capacity can lead to the persistent occurrence of herbicide-resistant weeds (Chen et al., 2023).

Tembotrione, a novel triketone herbicide (HRAC Group 27), was developed by Bayer CropScience for weed control in maize fields. Tembotrione disrupts carotenoids biosynthesis by inhibiting the enzyme HPPD in weeds, and it displays great efficacy in controlling both broadleaf and grassy weeds that are currently challenging to manage (Williams and Pataky, 2008). Despite its rapid increase in use overseas during the past decade, it was just registered for weed control in maize fields in China on 31 December 2021 (Gao et al., 2024). Since the exclusive dependence on the same MOA herbicides is major contributor to the rapid evolution of herbicide resistance (Norsworthy et al., 2012), it is essential to evaluate the potential risk of tembotrione resistance development in problematic weeds.

The variance in genetic diversity and sensitivity is connected to the potential development of herbicide resistance in a specific species (Moss, 2017). Of these, the variation in susceptibility indicated the differences in the response of each weed species to a certain herbicide among its populations, and it is mainly investigated via the baseline susceptibility test. A baseline test aims to determine the natural variation in susceptibility of a particular weed species to a certain herbicide in a specific period or area. It generally requires substantial efforts and resources. Notably, by using the populations of a certain weed species collected from various geographical locations and temporal periods, the baseline test can also estimate the potential risk of resistance development (Espeby et al., 2011).

In recent years, farmers in the lower-middle reaches of the Yangtze River basin, especially in Anhui Province, have encountered growing challenges in the effective management of *Echinochloa* spp. using common chemical herbicides, such as metamifop and penoxsulam, at their respective recommended field rates (RFRs) (Chen et al., 2016; Li et al., 2023). Numerous studies have gathered relevant of *Echinochloa* spp. resistant populations and investigated their resistance distribution; however, most of these studies analyzed only a limited number of populations from specific locations and focused on single MOA herbicides. Consequently, the status and extent of resistance to commonly used herbicides with different MOAs within a given region require further investigation. Moreover, considering the variations in weed species in maize fields and the accompanied herbicide application history, it becomes imperative to establish the baseline susceptibility of problematic weeds in China to newly registered herbicides and estimate the potential risk of resistance development. Therefore, this current study aims to (i) determining the distributions and resistance levels of diverse *Echinochloa* spp. populations collected across Anhui province to two most commonly used herbicides, metamifop and penoxsulam, (ii) exploring possible TSR mechanisms in metamifop- and penoxsulam-resistance populations, (iii) determining the natural variation in susceptibility among these diverse populations to the HPPD inhibitor tembotrione.

MATERIALS AND METHODS

Plant materials and growth conditions

From 2020 to 2021, a total of 85 *Echinochloa* spp. populations comprised 23 *E. crusgalli* var. *mitis*, 19 *E. glabrescens*, 9 *E. caudata*, 7 *E. oryzoides*, 7 *E. crusgalli* var. *zelayensis*, 6 *E. colonum* and 14 *E. hispidula* which had not been exposed to tembotrione were collected from different areas covering rice (*Oryza sativa* L.) and maize (*Zea mays* L.) fields in Anhui Province, China (Table 1, Figure 1). The mature seeds of each population were harvested from over 200 plants at each site and pooled into one sample. After being air dried, all the seeds were stored at 4 °C until use.

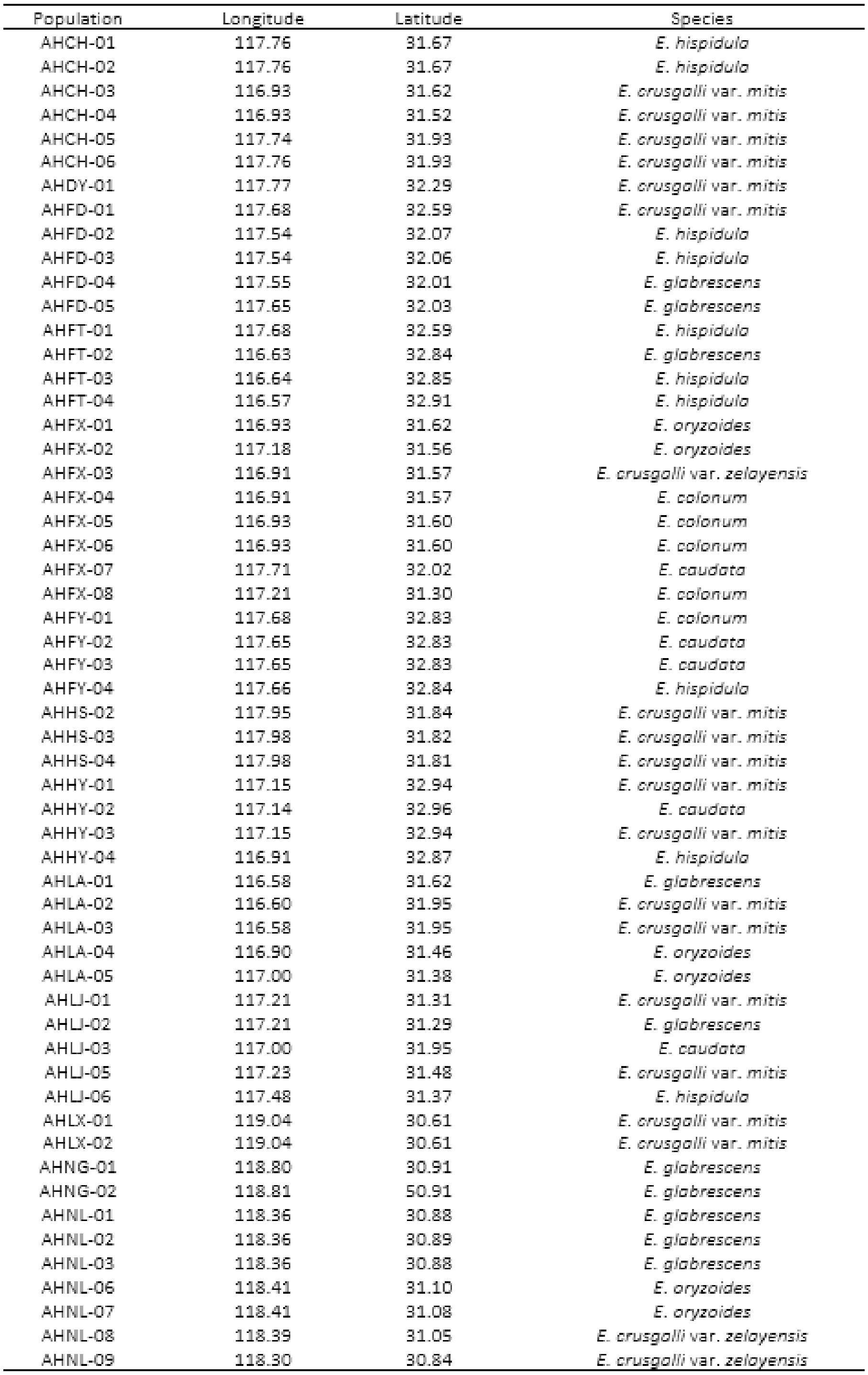
Seeds were chosen at random from each population and germinated in Petri dishes containing distilled water. After germination, the seedlings with shoots 1 cm in length were sown into 12 cm-diameter plastic pots containing loam soil (10 seedlings per pot) and grown to the 2- to 3-leaf stage in a controlled glasshouse (natural light, 35 °C/25 °C, ∼ 75% relative humidity). All pots were watered and re-arranged regularly. When the seedlings reached the 3- and 4-leaf stage, they were thinned to 6 individuals per pot that were evenly sized and continually grown.

Initial resistance screening with single herbicide dose

Two herbicides, including an ACCase inhibitor metamifop ((2*R*)-2-[4-[(6-chloro-1,3-benzoxazol-2-yl)oxy]phenoxy]-*N*-(2-fluorophenyl)-*N*-methylpropanamide; 10% EC, FMC, Suzhou, China) and an ALS inhibitor penoxsulam (2-(2,2-difluoroethoxy)-*N*-(5,8-dimethoxy[1,2,4]triazolo[1,5-c]pyrimidin-2-yl)-6-(trifluoromethyl)benzenesulfonamide; 25 g L^{-1} OD, Corteva, Shanghai, China), were selected to test the herbicide susceptibility in the *Echinochloa* spp. populations. When weed seedlings reached the 3- to 4-leaf stage, they were foliar treated with different herbicides at their respective recommended field rates (RFRs). For each population, a total of 54 plants were used for herbicide resistance screening. Of them, 18 plants were sprayed with metamifop at 120 g active ingredient (ai) ha^{-1}, 18 plants were sprayed with penoxsulam at 30 g ai ha^{-1}, and the remaining 18 plants sprayed with water served as the untreated control. Both herbicides and water were sprayed using a laboratory cabinet sprayer (3WP-2000, Nanjing Mechanization Research Institute of the Ministry of Agriculture, Nanjing, China) equipped with a flat-fan nozzle, which delivered 450 L ha^{-1} water at 275 kPa and 0.5 m s^{-1} (Zhao et al., 2022). After treatment, all the weed were grown for another 3 wk under conditions mentioned earlier. At 21 d after treatment (DAT), the above-ground biomass was determined for each treatment and expressed as a percentage of the untreated control. The experiment was repeated twice with each treatment containing three biological replicates.

In this present study, metamifop and penoxsulam treatments at their RFRs caused the most significant reductions of 97.80% and 98.30% in the fresh weights of the populations AHXZ-01 and AHLJ-03, respectively. Therefore, AHXZ-01 and AHLJ-03 were separately used as the standard susceptible (SS) populations to metamifop and penoxsulam for resistance classification. According to the R resistance rating system proposed by Moss et al. (2007), the susceptibility of *Echinochloa* spp. populations to metamifop and penoxsulam were divided into four resistance categories (S, R?, RR and RRR), in which S is the susceptible population, and the fresh weight inhibition rate ranges from 9 ×SS/10 to SS; R? means that the growth of populations could be effectively inhibited by herbicides, and its fresh weight inhibition rate ranges from 4× SS/5 to 9× SS/10; RR means that the population was proven to be resistant to herbicides, and its fresh weight inhibition rate ranges from 2× SS/5 to 4× SS/5; RRR means high-level herbicide resistance verified and its fresh weight inhibition rate ranges from 0 to 4× SS/5.

Table 1. The geographical position of 85 *Echinochloa* spp. populations.



Population	Longitude	Latitude	Species
AHCH-01	117.76	31.67	*E. hispidula*
AHCH-02	117.76	31.67	*E. hispidula*
AHCH-03	116.93	31.62	*E. crusgalli* var. *mitis*
AHCH-04	116.93	31.52	*E. crusgalli* var. *mitis*
AHCH-05	117.74	31.93	*E. crusgalli* var. *mitis*
AHCH-06	117.76	31.93	*E. crusgalli* var. *mitis*
AHDY-01	117.77	32.29	*E. crusgalli* var. *mitis*
AHFD-01	117.68	32.59	*E. crusgalli* var. *mitis*
AHFD-02	117.54	32.07	*E. hispidula*
AHFD-03	117.54	32.06	*E. hispidula*
AHFD-04	117.55	32.01	*E. glabrescens*
AHFD-05	117.65	32.03	*E. glabrescens*
AHFT-01	117.68	32.59	*E. hispidula*
AHFT-02	116.63	32.84	*E. glabrescens*
AHFT-03	116.64	32.85	*E. hispidula*
AHFT-04	116.57	32.91	*E. hispidula*
AHFX-01	116.93	31.62	*E. oryzoides*
AHFX-02	117.18	31.56	*E. oryzoides*
AHFX-03	116.91	31.57	*E. crusgalli* var. *zelayensis*
AHFX-04	116.91	31.57	*E. colonum*
AHFX-05	116.93	31.60	*E. colonum*
AHFX-06	116.93	31.60	*E. colonum*
AHFX-07	117.71	32.02	*E. caudata*
AHFX-08	117.21	31.30	*E. colonum*
AHFY-01	117.68	32.83	*E. colonum*
AHFY-02	117.65	32.83	*E. caudata*
AHFY-03	117.65	32.83	*E. caudata*
AHFY-04	117.66	32.84	*E. hispidula*
AHHS-02	117.95	31.84	*E. crusgalli* var. *mitis*
AHHS-03	117.98	31.82	*E. crusgalli* var. *mitis*
AHHS-04	117.98	31.81	*E. crusgalli* var. *mitis*
AHHY-01	117.15	32.94	*E. crusgalli* var. *mitis*
AHHY-02	117.14	32.96	*E. caudata*
AHHY-03	117.15	32.94	*E. crusgalli* var. *mitis*
AHHY-04	116.91	32.87	*E. hispidula*
AHLA-01	116.58	31.62	*E. glabrescens*
AHLA-02	116.60	31.95	*E. crusgalli* var. *mitis*
AHLA-03	116.58	31.95	*E. crusgalli* var. *mitis*
AHLA-04	116.90	31.46	*E. oryzoides*
AHLA-05	117.00	31.38	*E. oryzoides*
AHLJ-01	117.21	31.31	*E. crusgalli* var. *mitis*
AHLJ-02	117.21	31.29	*E. glabrescens*
AHLJ-03	117.00	31.95	*E. caudata*
AHLJ-05	117.23	31.48	*E. crusgalli* var. *mitis*
AHLJ-06	117.48	31.37	*E. hispidula*
AHLX-01	119.04	30.61	*E. crusgalli* var. *mitis*
AHLX-02	119.04	30.61	*E. crusgalli* var. *mitis*
AHNG-01	118.80	30.91	*E. glabrescens*
AHNG-02	118.81	50.91	*E. glabrescens*
AHNL-01	118.36	30.88	*E. glabrescens*
AHNL-02	118.36	30.89	*E. glabrescens*
AHNL-03	118.36	30.88	*E. glabrescens*
AHNL-06	118.41	31.10	*E. oryzoides*
AHNL-07	118.41	31.08	*E. oryzoides*
AHNL-08	118.39	31.05	*E. crusgalli* var. *zelayensis*
AHNL-09	118.30	30.84	*E. crusgalli* var. *zelayensis*

Cont. Table 1.

AHPJ-01	116.55	31.95	*E. hispidula*
AHPJ-02	116.54	31.90	*E. hispidula*
AHQS-01	117.34	32.35	*E. crusgalli* var. *mitis*
AHQS-02	117.65	32.99	*E. crusgalli* var. *mitis*
AHQS-03	116.58	30.67	*E. crusgalli* var. *mitis*
AHQS-04	116.58	30.68	*E. caudata*
AHQS-05	116.62	30.78	*E. caudata*
AHQS-06	116.57	30.52	*E. caudata*
AHSC-01	117.01	31.54	*E. crusgalli* var. *zelayensis*
AHSC-02	117.00	31.38	*E. crusgalli* var. *zelayensis*
AHSC-03	116.63	30.78	*E. glabrescens*
AHSC-04	116.63	30.78	*E. glabrescens*
AHSC-05	116.89	31.47	*E. crusgalli* var. *mitis*
AHSC-06	117.05	31.47	*E. caudata*
AHSS-01	116.11	30.25	*E. crusgalli* var. *mitis*
AHSS-02	116.12	30.24	*E. crusgalli* var. *mitis*
AHSX-01	116.81	31.95	*E. hispidula*
AHSX-02	116.78	31.99	*E. glabrescens*
AHSX-03	116.76	32.00	*E. glabrescens*
AHSX-04	116.61	32.08	*E. hispidula*
AHTC-01	119.09	32.73	*E. colonum*
AHTC-02	118.54	32.88	*E. glabrescens*
AHWJ-01	116.61	30.10	*E. glabrescens*
AHWZ-01	118.36	30.88	*E. crusgalli* var. *zelayensis*
AHXZ-01	118.67	30.88	*E. glabrescens*
AHXZ-02	118.51	31.12	*E. oryzoides*
AHXZ-03	118.35	30.88	*E. crusgalli* var. *zelayensis*
AHYA-01	118.04	31.98	*E. glabrescens*
AHYA-02	118.00	31.03	*E. glabrescens*

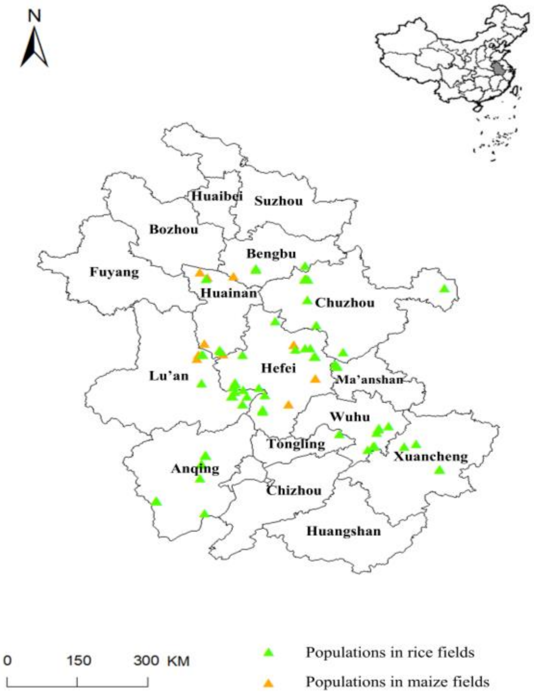


Figure 1. Distribution of 85 *Echinochloa* Spp. populations collected from rice and maize fields in Anhui Province, China.

Target gene amplification and sequencing

To investigate the potential TSR mechanisms to metamifop and penoxsulam conferred by target-site mutations, both the metamifop- and penoxsulam-resistant populations were selected for *ACCase* and/or *ALS* gene sequencing, respectively. Ten plants were randomly selected from each population and were grown to the 3-to 4-leaf stage. About 50 mg young shoot tissue was then harvested from the leaves of each individual plant and immediately frozen in liquid nitrogen. Genomic DNA was extracted from each sample according to the manufactures of the NuClean Plant Genomic DNA Kit (CWBIO, Shanghai, China). For *ACCase* gene, a pair of primers (Forward: 5′-ATCTTGCTTCCGTGTTGGGT-3′, Reverse: 5′-CCTTGGAGTTTTGCTTTCAG-3′) reported by Yang et al. (2021), covering all seven amino acid positions which can mutate to confer resistance to ACCase inhibitors, were used for amplification from both resistant and susceptible plant samples. In addition, the complete coding sequences of *ALS* containing all nine amino acid positions which can mutate to confer resistance to ALS inhibitors were amplified using the primers (Forward: 5′-GTCATCGCCAACCACCTCT-3′, Reverse: 5′-CGACTCACCAACAAGACGC-3′) reported by Fang et al. (2019). Polymerase chain reaction (PCR) was performed with 50 µL reaction system which contained 2.5 ng of template DNA, 2 µL each primer (10 µM), 25 µL 2× Es Taq Master Mix (CWBIO, Beijing, China) and added ddH_2O to 50 µL. The PCR conditions were as follows: Denaturation 94 °C for 2 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 58 to 62 °C, 72 °C for 49 s and with the final extension step of 2 min at 72 °C. The PCR products were checked on 1.0% agarose gel in 1× TAE buffer and then sequenced by Tsingke Biotech (Beijing, China). The sequences obtained from the resistant and susceptible populations were aligned and compared using DNAMAN v.6.0 software (Lynnon Biosoft, Quebec, Canada).

Dose-response experiments to metamifop and penoxsulam

According to the result of single-dose testing, all the R?, RR and RRR populations were chose to investigate the resistance level to metamifop and penoxsulam by whole-plant dose-response assays. Weed seedlings were grown to the 3- to 4-leaf stage and then spread the series doses of metamifop (1.5, 4.4, 13.3, 40 and 120 g ai ha^{-1} for S population, and 13.3, 40, 120, 360 and 1080 g ai ha^{-1} for R population) and penoxsulam (0.3, 1.1, 3.3, 10 and 30 g ai ha^{-1} for S population, and 3.3, 10, 30, 90 and 270 g ai ha^{-1} for R population). The above-ground fresh weight of the R and S plants at 21 DAT was recorded and the whole experiment was repeated twice, with each treatment containing three biological replicates.

Dose-response experiments to tembotrione

To determine the sensitivity of each population and investigated the baseline sensitivity to the HPPD inhibitor tembotrione, whole-plant dose-response assays were conducted. Weed seedlings were grown to the 3- to 4-leaf stage and sprayed with the series doses of tembotrione. Based on the preliminary experimental results (data not shown), technical tembotrione (2-[2-chloro-4-methylsulfonyl-3-(2,2,2-trifluoroethoxymethyl)benzoyl]cyclohexane-1,3-dione; 94.1%, Anhui Jiuyi Agriculture Co., Ltd., Hefei, China) dissolved and diluted with a 0.1% aqueous solution of Tween-80 (Solarbio Life Sciences, Beijing, China) was used at 0, 1.23, 3.70, 11.11, 33.33, 100, and 300 g ai ha^{-1} for different populations. The above-ground fresh weight of the R and S plants at 21 DAT was recorded and the whole experiment was repeated twice, with each treatment containing three biological replicates.

Data analysis

The datasets from the same treatment in the repeated experiments (runs) were firstly subjected to ANOVA in SPSS v19.0 (IBM, Armonk, New York, USA). Because nonsignificant difference ($P > 0.05$) was detected between the repeated runs of a given experiment, the data from the same treatment were pooled across runs and fitted to a four-parameter log-logistic curve (Equation 1) in SigmaPlot v. 14.0 (Systat Software, San Jose, California, USA):

$$y = C + \frac{D - C}{1 + \left(\frac{x}{\mathrm{GR}_{50}}\right)^{b}} \qquad [1]$$

where y is the response at the herbicide dose x, C is the lower limit of the response, D is the upper limit of the response, b is the slope at the herbicide dose causing a 50% growth reduction (GR_{50}). The baseline sensitivity

($GR_{50}b$) was determined by calculating the average GR_{50} values across all examined populations. The SI_{50} value, sensitivity index at 50% growth reduction, was determined by dividing the greatest GR_{50} value by the smallest GR_{50} value, and the $SI_{50}b$ value was calculated by dividing the greatest GR_{50} value by the $GR_{50}b$ value (Escorial et al., 2019). The resistance index (RI) based on the GR_{50} values was calculated by dividing the resistant population by the susceptible population.

To evaluate the range of susceptibility distributions, the skewness (β_3) and kurtosis (β_4) were also analyzed via Equations 2 and 3, respectively, based on the GR_{50} values of all populations.

$$\beta_3=\sum\frac{(x_i-\bar{x})^3}{ns^3} \qquad [2]$$

$$\beta_4=\sum\frac{(x_i-\bar{x})^4}{ns^4} \qquad [3]$$

where x_i is the i^{th} GR_{50} value, $\bar{x}$ is the mean of GR_{50} values, n is the number of values, and s is the standard deviation.

RESULTS AND DISCUSSION

Single-dose testing for different *Echinochloa* spp. populations

A total of 85 populations of *Echinochloa* spp. collected from rice and maize fields across Anhui Province, China, were tested for their susceptibility to metamifop and penoxsulam, respectively. Populations AHXZ-01 and AHLJ-03 with fresh weight reductions of 97.80% and 98.30% to metamifop and penoxsulam were separately used as the SS populations for resistance classification.

At 21 metamifop DAT, 41 of the total 85 populations were susceptible to metamifop and showing fresh weight reductions ranging from 88.30% to 97.80%, 27 populations were classified as R? with fresh weight reductions ranging from 78.35% to 87.96%, 15 populations were classified as RR with fresh weight reduction ranging from 40.69% to 77.78%, and two populations were classified as RRR with fresh weight reduction of 2.70% and 36.67% (Table 2, Figure 2A).

Similarly, at 21 d after penoxsulam treatment, 22 of the total 85 populations were susceptible to penoxsulam and showing fresh weight reductions ranging from 88.50% to 98.30%, 22 populations were classified as R? with fresh weight reductions ranging from 78.80% to 88.10%, 22 populations were classified as RR with fresh weight reductions ranging from 39.40% to 78.30%, and 19 populations were classified as RRR with fresh weight reductions ranged from 3.18% to 36.99% (Table 2, Figure 2B).

Anhui Province, located in the middle and lower reaches of the Yangtze River, stands as a main region for rice and maize production in China. *Echinochloa* spp., recognized as the most pernicious weeds in rice and maize fields, have developed resistance to commonly employed herbicides (Hwang et al., 2023). In recent years, there has been frequent identification of metamifop and penoxsulam resistance in distinct populations of *Echinochloa* spp. (Li et al., 2023; Zhang et al., 2023). However, these studies primarily focused on individual herbicides, often examining only one or a few populations from specific locales. Consequently, there is currently no detailed characterization of the distributions and resistance patterns of diverse *Echinochloa* spp. populations to metamifop and penoxsulam across Anhui Province. In this study, a total of 85 populations of *Echinochloa* spp. was meticulously collected, encompassing Anhui's major rice and maize production areas. Among them, 44 and 61 populations were identified to have developed varying degrees of resistance to metamifop and penoxsulam. Remarkably, 30 populations have evolved multiple resistances to both metamifop and penoxsulam. This underscores the widespread distribution of *Echinochloa* spp. resistance in Anhui's crop fields, with penoxsulam resistance appearing more severe. This emphasizes the urgent need to introduce novel and highly efficient herbicides and implement integrated weed management (IPM) practices.

Table 2. Susceptibility to two herbicides and resistance mutations detected in different *Echinochloa* spp. populations tested in this study. RFW: Reduction in fresh weight; RI: resistance index. [a]Sensitivity to metamifop and penoxsulam was divided into four grades. S: susceptible population; R?: growth of populations could effectively inhibit by herbicides; RR: populations which resistance have determined; RRR: high resistance level populations to herbicide.

	Metamifop					Penoxsulam				
Population	RFW (SE)	Biotype[a]	Mutation	GR_{50} (SE)	RI	RFW (SE)[a]	Biotype[a]	Mutation	GR_{50} (SE)	RI
	%			g ai ha^{-1}		%			g ai ha^{-1}	
AHCH-01	97.20 (1.30)	S	-	-	-	95.70 (9.0)	S	-	-	-
AHCH-02	90.00 (2.00)	S	-	-	-	97.00 (1.0)	S	-	-	-
AHCH-03	86.90 (2.30)	R?	-	48.64 (10.48)	2.64	97.70 (0.7)	S	-	-	-
AHCH-04	96.80 (1.30)	S	-	-	-	96.00 (1.2)	S	-	-	-
AHCH-05	84.21 (1.07)	R?	-	57.28 (4.16)	3.11	84.21 (3.48)	R?	-	16.08 (1.93)	3.50
AHCH-06	85.14 (2.86)	R?	-	54.31 (34.50)	2.95	39.92 (5.51)	RR	-	40.16 (8.94)	8.73
AHDY-01	88.30 (2.10)	S	-	-	-	89.40 (10.0)	S	-	-	-
AHFD-01	86.40 (2.80)	R?	-	48.94 (2.15)	2.66	78.30 (14.0)	RR	-	23.98 (15.04)	5.21
AHFD-02	91.90 (1.14)	S	-	-	-	13.08 (7.19)	RRR	-	62.87 (13.40)	13.67
AHFD-03	92.53 (1.24)	S	-	-	-	85.44 (1.18)	R?	-	14.91 (1.12)	3.24
AHFD-04	36.67 (21.37)	RRR	-	139.08 (7.10)	7.55	20.48 (5.17)	RRR	Trp-574-Leu	49.45 (7.32)	10.75
AHFD-05	40.69 (6.70)	RR	-	126.85 (22.19)	6.89	30.84 (15.17)	RRR	-	43.57 (25.72)	9.47
AHFT-01	96.90 (0.90)	S	-	-	-	97.10 (1.0)	S	-	-	-
AHFT-02	83.53 (1.43)	R?	-	64.06 (8.41)	3.48	33.73 (1.74)	RRR	-	42.44 (4.90)	9.23
AHFT-03	91.16 (1.96)	S	-	-	-	76.52 (9.74)	RR	-	27.39 (5.66)	5.95
AHFT-04	91.59 (3.13)	S	-	-	-	76.70 (8.95)	RR	-	24.59 (3.02)	5.35
AHFX-01	88.40 (4.70)	S	-	-	-	78.80 (6.0)	R?	-	22.43 (3.98)	4.88
AHFX-02	96.40 (1.90)	S	-	-	-	76.80 (5.0)	RR	-	24.28 (5.33)	5.28
AHFX-03	77.00 (0.25)	RR	-	94.38 (10.99)	5.12	87.00 (1.0)	R?	-	11.89 (0.61)	2.58
AHFX-04	89.00 (3.30)	S	-	-	-	85.00 (1.0)	R?	-	14.92 (3.23)	3.24
AHFX-05	91.00 (9.60)	S	-	-	-	64.90 (1.0)	RR	-	29.38 (10.96)	6.39
AHFX-06	80.10 (4.20)	R?	-	79.87 (13.06)	4.34	96.40 (0.8)	S	-	-	-
AHFX-07	75.40 (8.60)	RR	-	110.36 (46.92)	5.99	96.60 (1.0)	S	-	-	-
AHFX-08	76.20 (4.30)	RR	-	101.95 (1.58)	5.53	98.10 (0.6)	S	-	-	-
AHFY-01	84.00 (2.60)	R?	-	63.92 (5.22)	3.47	98.00 (0.4)	S	-	-	-
AHFY-02	76.80 (4.80)	RR	-	97.70 (5.39)	5.30	97.20 (0.6)	S	-	-	-
AHFY-03	81.30 (8.90)	R?	-	71.06 (4.61)	3.86	97.30 (1.0)	S	-	-	-
AHFY-04	91.74 (1.79)	S	-	-	-	55.37 (7.16)	RR	-	31.13 (4.42)	6.77
AHHS-02	89.03 (6.42)	S	-	-	-	18.63 (2.82)	RRR	-	51.59 (11.72)	11.22
AHHS-03	82.35 (0.60)	R?	-	67.99 (9.67)	3.69	83.09 (4.41)	R?	-	18.58 (0.27)	4.04
AHHS-04	60.39 (17.72)	RR	-	121.08 (11.94)	6.57	50.43 (14.43)	RR	-	35.87 (8.53)	7.80
AHHY-01	84.20 (4.60)	R?	-	60.35 (4.83)	3.28	81.60 (9.0)	R?	-	20.94 (1.63)	4.55
AHHY-02	76.00 (7.90)	RR	-	105.42 (11.49)	5.72	97.20 (1.1)	S	-	-	-
AHHY-03	82.70 (8.90)	R?	-	67.39 (4.15)	3.66	82.10 (4.0)	R?	-	20.10 (0.21)	4.37
AHHY-04	94.20 (2.05)	S	-	-	-	85.51 (2.51)	R?	-	14.28 (1.72)	3.10
AHLA-01	84.10 (0.80)	R?	-	62.12 (2.21)	3.37	49.90 (13.0)	RR	-	37.15 (7.57)	8.08
AHLA-02	87.00 (3.60)	R?	-	39.98 (8.84)	2.17	53.40 (2.0)	RR	-	32.54 (3.026)	7.07
AHLA-03	85.90 (9.80)	R?	-	53.33 (2.03)	2.90	24.70 (9.0)	RRR	-	48.63 (18.10)	10.57
AHLA-04	87.00 (1.20)	R?	-	44.27 (14.33)	2.40	52.20 (8.0)	RR	-	32.85 (3.14)	7.14
AHLA-05	76.60 (8.60)	RR	-	100.32 (8.76)	5.45	94.90 (1.0)	S	-	-	-
AHLI-01	96.90 (1.80)	S	-	-	-	87.70 (6.0)	R?	-	11.67 (1.69)	2.54
AHLI-02	2.70 (0.70)	RRR	-	198.85 (24.61)	10.80	89.50 (3.5)	S	-	-	-
AHLI-03	97.70 (1.10)	S	-	-	-	98.30 (1.0)	SS	-	4.60 (0.28)	-
AHLI-05	63.86 (5.92)	RR	-	118.88 (34.48)	6.45	60.11 (5.21)	RR	-	29.61 (22.19)	6.44
AHLI-06	89.39 (5.84)	S	-	-	-	55.30 (11.60)	RR	-	31.37 (6.09)	6.82
AHLX-01	93.20 (2.10)	S	-	-	-	90.00 (2.0)	S	-	-	-
AHLX-02	97.70 (1.10)	S	-	-	-	58.20 (13.5)	RR	-	30.26 (7.11)	6.58
AHNG-01	96.40 (2.50)	S	-	-	-	75.70 (5.6)	RR	-	27.56 (1.68)	5.99
AHNG-02	90.30 (0.30)	S	-	-	-	84.70 (2.1)	R?	-	15.19 (1.14)	3.30
AHNL-01	93.10 (1.20)	S	-	-	-	39.40 (1.0)	RR	-	40.81 (5.31)	8.87
AHNL-02	97.30 (4.60)	S	-	-	-	61.10 (9.0)	RR	-	29.49 (9.84)	6.41
AHNL-03	80.70 (3.40)	R?	-	75.81 (4.69)	4.12	46.60 (10.0)	RR	-	37.48 (10.66)	8.15
AHNL-06	90.62 (0.92)	S	-	-	-	51.60 (9.20)	RR	Trp-574-Leu	33.26 (4.66)	7.23
AHNL-07	81.58 (3.72)	R?	-	70.03 (11.72)	3.80	85.96 (5.48)	R?	-	13.96 (0.38)	3.03
AHNL-08	96.62 (2.73)	S	-	-	-	80.72 (7.03)	R?	-	21.61 (3.33)	4.70
AHNL-09	91.03 (3.63)	S	-	-	-	82.69 (6.93)	R?	-	19.04 (0.23)	4.14
AHPJ-01	94.31 (1.15)	S	-	-	-	83.74 (6.14)	R?	-	18.18 (11.33)	3.95
AHPJ-02	93.59 (3.63)	S	-	-	-	85.90 (4.44)	R?	-	14.13 (2.77)	3.07
AHQS-01	76.70 (3.90)	RR	-	99.04 (7.02)	5.38	52.90 (1.0)	RR	-	32.76 (9.30)	7.12
AHQS-02	78.40 (7.70)	R?	-	84.61 (16.64)	4.59	76.60 (6.2)	RR	-	26.11 (1.67)	5.68
AHQS-03	67.92 (3.58)	RR	-	112.29 (23.06)	6.10	14.17 (14.81)	RRR	-	56.49 (19.36)	12.28
AHQS-04	78.35 (8.27)	R?	-	85.45 (7.52)	4.64	4.84 (3.24)	RRR	-	70.06 (34.61)	15.23
AHQS-05	78.57 (3.89)	R?	-	83.54 (2.745)	4.54	86.23 (4.70)	R?	-	12.55 (6.15)	2.73
AHQS-06	95.04 (2.01)	S	-	-	-	84.78 (3.77)	R?	-	15.02 (3.77)	3.27
AHSC-01	81.50 (4.00)	R?	-	70.79 (23.14)	3.84	96.40 (1.5)	S	-	-	-
AHSC-02	97.10 (1.60)	S	-	-	-	96.60 (1.0)	S	-	-	-
AHSC-03	92.40 (0.80)	S	-	-	-	66.60 (4.0)	RR	-	28.85 (4.66)	6.27
AHSC-04	83.40 (1.60)	R?	-	65.30 (6.95)	3.55	95.80 (1.2)	S	-	-	-
AHSC-05	77.02 (2.52)	RR	-	93.26 (6.80)	5.06	18.60 (4.54)	RRR	-	53.45 (5.90)	11.62
AHSC-06	77.78 (8.48)	RR	-	92.33 (1.40)	5.01	18.52 (5.66)	RRR	-	54.81 (1.16)	11.92
AHSS-01	67.25 (17.13)	RR	-	114.75 (37.22)	6.23	10.66 (11.98)	RRR	-	63.88 (18.47)	13.89
AHSS-02	45.88 (6.72)	RR	-	123.85 (12.80)	6.72	7.84 (9.80)	RRR	-	65.29 (9.57)	14.19
AHSX-01	88.98 (4.48)	S	-	-	-	22.31 (6.15)	RRR	-	49.34 (12.42)	10.73
AHSX-02	81.62 (4.21)	R?	-	68.50 (19.53)	3.72	88.65 (1.17)	S	-	9.46 (3.54)	2.06
AHSX-03	87.96 (1.99)	R?	-	38.21 (4.83)	2.07	36.99 (27.70)	RRR	-	40.91 (4.73)	8.89
AHSX-04	91.08 (1.38)	S	-	-	-	3.18 (16.67)	RRR	Trp-574-Leu	73.80 (36.53)	16.04
AHTC-01	82.80 (4.70)	R?	-	66.18 (11.24)	3.59	5.10 (22.0)	RRR	Trp-574-Leu	69.65 (55.34)	15.14
AHTC-02	80.90 (1.00)	R?	-	74.22 (11.51)	4.03	88.50 (1.1)	S	-	9.81 (1.91)	2.13
AHWJ-01	91.67 (1.68)	S	-	-	-	85.61 (1.52)	R?	-	14.20 (4.16)	3.09
AHWZ-01	97.40 (3.20)	S	-	-	-	96.70 (1.2)	S	-	-	-
AHXZ-01	97.80 (0.50)	SS	-	18.42 (2.37)	1	97.20 (1.0)	S	-	-	-
AHXZ-02	96.40 (7.00)	S	-	-	-	18.80 (15.6)	RRR	Trp-574-Leu	51.53 (29.99)	11.20
AHXZ-03	96.80 (0.70)	S	-	-	-	88.10 (3.2)	R?	-	9.99 (1.66)	2.17
AHYA-01	91.84 (1.76)	S	-	-	-	34.75 (11.74)	RRR	-	41.72 (15.71)	9.07
AHYA-02	80.57 (4.72)	R?	-	78.56 (4.86)	4.26	27.37 (14.59)	RRR	-	44.99 (8.53)	9.78

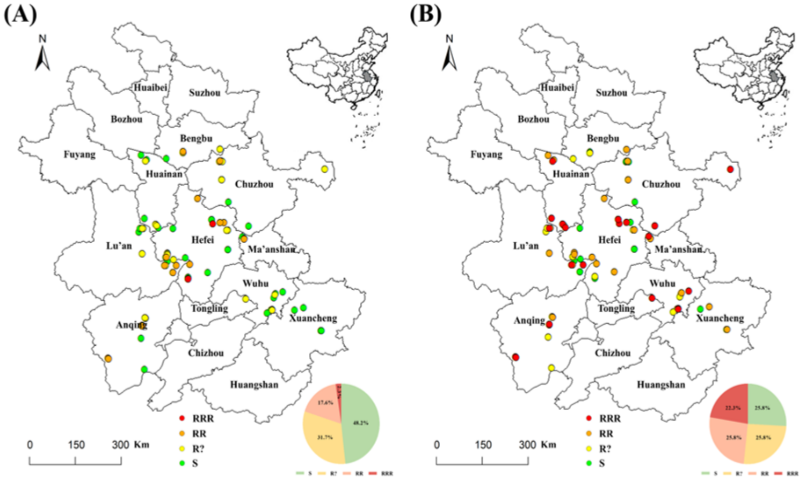


Figure 2. The susceptible of 85 *Echinochloa* spp. populations to (A) metamifop and (B) penoxsulam. Four resistance categories (S, R?, RR, RRR) were determined based on the susceptibility of *Echinochloa* spp. populations to metamifop and penoxsulam. S: Population is susceptible; R?: growth of populations could be effectively inhibited by herbicides; RR: resistance has been determined, which probably results in a reduction in the performance of herbicides; RRR: high resistance level to metamifop and penoxsulam.

Sequencing of *ACCase* and *ALS* genes

For populations classed as SS, R?, RR, and RRR to metamifop and penoxsulam, 1254 and 2353 bp fragments of *ACCase* and *ALS* genes were each amplified from their respective plants. Comparison of the *ACCase* genes between the SS and metamifop-resistant plants revealed no resistance amino acid substitution (AAS) in any resistant populations (Table 2). However, a single AAS of Trp-to-Leu at codon position 574 of *ALS* were identified in five penoxsulam-resistant populations, including AHFD-04, AHNL-06, AHSX-04, AHTC-01 and AHXZ-02 (Table 2). No known/unknown resistance mutations were detected in the *ALS* genes of the remaining penoxsulam-resistant populations (data not shown).

Target-site mutation is one of the main reasons for weed resistance, which can generally reduce the affinity of target enzyme to herbicides through altering the conformations of the target enzymes (Sen et al., 2021). To date, nine and seven codon positions have been identified in the *ALS* and *ACCase* genes, respectively, to be able to mutate to confer resistance to corresponding inhibitors (Fang et al., 2022; Jiang et al., 2022; Yin et al., 2023). Many weed species have been reported to exhibit high-level resistance to ACCase and ALS inhibitors through target gene mutations, such as *B. syzigachne* (Yin et al., 2023), shortawn foxtail (*Alopecurus aequalis* Sobol.) (Guo et al., 2018), Japanese foxtail (*Alopecurus japonicus* Steud.) (Bi et al., 2016), and rigid ryegrass (*Lolium rigidum* Gaudich.) (Anthimidou et al., 2020). For *Echinochloa* spp. specially, Iwakami et al. (2023) identified various AASs in different *E. crus-galli* populations, such as Trp-1999-Cys, Trp-2027-Cys, Trp-2027-Ser, and Ile-2041-Asn, which conferred high resistance levels to ACCase inhibitors. Feng et al. (2022) identified two *E. crus-galli* populations showing 17- and 3-folds resistance to penoxsulam, respectively, and target gene sequencing revealed a Trp-574-Leu mutation in their *ALS* genes. In this research, we did not detect any resistance mutations in *ACCase* genes of the metamifop-resistant populations, but identified a Trp-574-Leu mutation in *ALS* genes of five penoxsulam-resistant populations, including AHFD-04, AHNL-06, AHSX-04, AHTC-01 and AHXZ-02. The identified mutations may be the major reasons for penoxsulam resistance observed in these populations. However, target-site mutations were not found in the most of resistant populations and the resistant populations were associated with NTSR mechanisms.

Herbicide resistance status of *Echinochloa* spp. to metamifop and penoxsulam

The resistance level of different populations to metamifop and penoxsulam were confirmed by whole-plant dose-response assays. The herbicide doses causing 50% growth reduction (GR_{50}) values of susceptible population AHXZ-01 was 18.42 g ai ha^{-1} to metamifop and AHLJ-03 was 4.60 g ai ha^{-1} to penoxsulam, respectively. Compared with AHXZ-01, the GR_{50} value of metamifop-resistant populations were ranging from 38.21 to 198.85 g ai ha^{-1} with resistance index (RI) ranging from 2.07 to 10.80. Furthermore, the GR_{50} values of penoxsulam-resistant populations were ranging from 9.46 to 73.80 g ai ha^{-1}, with RI ranging from 2.06 to 16.04 (Table 2).

In recent years, ACCase and ALS resistance have been reported in various weed species. Yin et al. (2024) identified 84% (31/37) *B. syzigachne* populations displayed high-level resistance to fenoxaprop-*P*-ethyl and 62% (23/37) of the mesosulfuron-methyl-resistant population exhibited low- and moderate-level resistance. This study confirmed 1.2% and 16.5% *Echinochloa* spp. populations evolved high-level resistance to metamifop and penoxsulam; 50.6% and 57.6% of them had evolved low- and moderate- resistance to metamifop and penoxsulam, respectively. These results indicated that the prevalence of penoxsulam-resistant *Echinochloa* spp. in Anhui Province being more severe than metamifop resistance populations.

Baseline sensitivity of diverse *Echinochloa* spp. populations to tembotrione

The GR_{50} values for all 85 populations to tembotrione were utilized to analyze their distribution frequency and investigate the potential risk of resistance development in diverse *Echinochloa* spp. Whole-plant dose-response experiments showed that the 85 tested populations were all susceptible to tembotrione with GR_{50} values varied from 6.34 to 43.94 g ai ha^{-1} (Figure 3), which were significantly lower than the registered RFRs of tembotrione (90 to 105 g ai ha^{-1}). The mean value was 17.96 g ai ha^{-1}, the median value was 16.72 g ai ha^{-1}, and the minimum and maximum values were 6.34 and 43.94 g ai ha^{-1}, respectively (Table 3). The frequency of the GR_{50} values was not normally distributed but distributed as a unimodal curve (Figure 4). Therefore, the mean value of 17.96 g ai ha^{-1} was defined as the baseline sensitivity (GR_{50}b) to tembotrione. Meanwhile, the GR_{50} distribution had a skew towards the sensitive end, indicating that all the tested populations collected from Anhui Province have a low risk of tembotrione resistance development (Wang et al., 2022). The SI_{50} (sensitivity index at 50% growth reduction) value exhibited a 6.93-fold difference between the highest and lowest GR_{50} values. The differences could be attributed to the variation between species or between ecotypes of a single species (Escorial et al., 2019). The sensitivity index was also calculated as the ratio between the GR_{50} values of the least sensitive population and the baseline GR_{50} value estimated for a range of susceptible populations (SI_{50}b). When the SI_{50}b value was used to measure the difference in tembotrione sensitivity among the *E. crus-galli* populations, the difference in tembotrione sensitivity declined from 6.93-fold (SI_{50}) to 2.45-fold (SI_{50}b). Therefore, the establishment of baseline sensitivity should be considered the natural variability in herbicide sensitivity among different weed populations, and in comparison, to the GR_{50} value of the most sensitive population, the GR_{50}b may be a more practical parameter for baseline sensitivity (Escorial et al., 2019; Wang et al., 2022).

For weeds that have not previously been treated with a certain herbicide or have never been exposed to herbicides with the same MOA, measuring the sensitivity of different populations to establish a baseline sensitivity of weeds to specific herbicides is a usefully tool for evaluating herbicide efficacy and developing weed resistance management (Espeby et al., 2011; Escorial et al., 2019). Wang et al. (2022) reported the baseline sensitivity of *E. crus-galli* to tripyrasulfone, with GR_{50}b 18.12 g ai ha^{-1} and SI_{50} 23.8. In the present study, frequency distribution of GR_{50} values to tembotrione followed a unimodal curve and towards the sensitive end with the GR_{50}b 17.96 g ai ha^{-1}, and the SI_{50} value was 6.93, suggesting that the potential risk development of resistance to tembotrione in *Echinochloa* spp. was lower than that to tripyrasulfone.

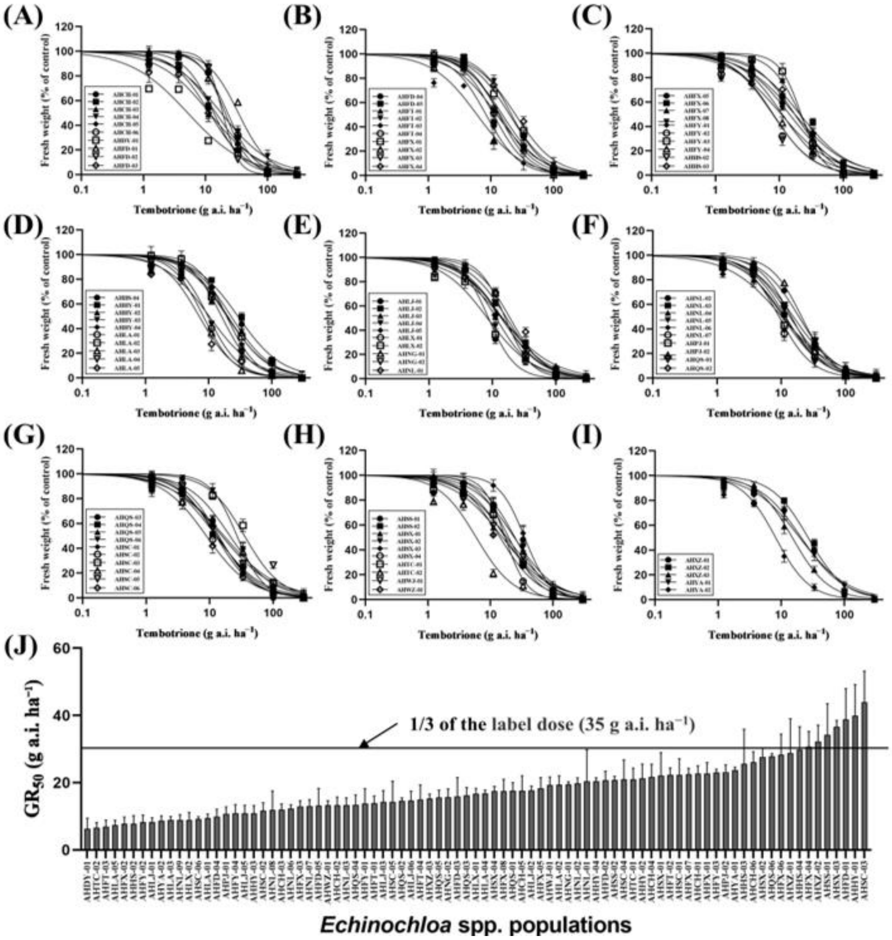


Figure 3. Dose response curve of *Echinochloa* spp. populations to tembotrione (A to I), the susceptibility of 85 *Echinochloa* spp. populations to tembotrione accession by dose-response experiments (J). Vertical bars represent the SE of the mean.

Table 3. The analysis of distribution and baseline sensitivity index based on the susceptibility of 85 *Echinochloa* spp. populations to tembotrione.

Index	*Echinochloa* spp. populations
Kurtosis	0.851
Skewness	0.974
Mean	17.96
Median	16.72
Maximum (A)	43.94
Minimum (B)	6.34
Baseline sensitivity index (A/B)	6.93

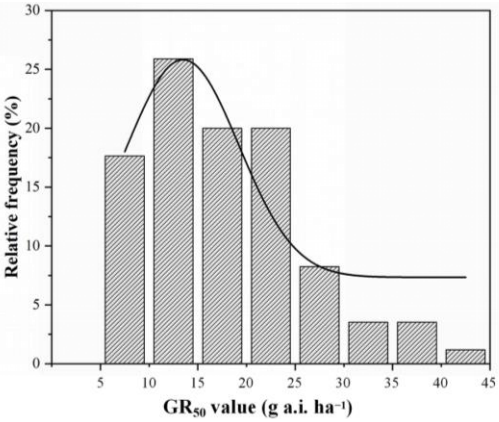


Figure 4. Distribution of the susceptibility of 85 *Echinochloa* spp. populations to tembotrione.

CONCLUSIONS

This study characterized the resistance distributions and extent of resistance in diverse *Echinochloa* spp. populations to metamifop and penoxsulam in Anhui Province, and explored the potential target gene mutations responsible for the observed resistance. The baseline sensitivity of these populations to the newly registered HPPD inhibitor tembotrione was also determined. The resistance of *Echinochloa* spp. to commonly used herbicides was found to be widely distributed and penoxsulam-resistant being more severe than metamifop-resistance populations across crop fields in Anhui Province. The baseline sensitivity test for *Echinochloa* spp. against tembotrione indicated that the risk of resistance to tembotrione was lower, which can be used as a potential alternative for achieving efficient weed control in Chinese maize fields. Nevertheless, these findings underscore the necessity for the implementation of integrated weed management practices to proactively delay the further development of resistance.

Author contributions

Conceptualization: M.L., W.H. Methodology: W.H., Y.L., Y.W., Q.L. Formal analysis: M.L., W.H. Investigation: W.H., Y.W., Z.W., X.Z., F.F. Writing—original draft preparation: W.H., Y.W., Q.L., M.L. Writing—review and editing: W.H., Z.W., N.Z. Supervision: M.L. Project administration: M.L. Funding acquisition: N.Z., M.L. All authors have read and agreed to the published version of the manuscript.

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