



Article

Survey Reveals Frequency of Multiple Resistance to Tribenuron-Methyl, Bensulfuron-Methyl and Halosulfuron-Methyl in Cleavers (*Galium aparine* L.)

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Abstract: Tribenuron-methyl-resistant (TmR) cleavers (*Galium aparine* L.) have been reported around the major winter wheat farming region in China. From 2017 to 2020, cleavers seeds were collected from wheat production fields across Jiangsu Province to evaluate the frequency and distribution of tribenuron-methyl-, bensulfuron-methyl- and halosulfuron-methyl-resistant cleavers, and to assess the frequency of multiple resistance. Here we report resistance frequency as percent resistance within a population, and resistance distribution as the percentage and locations of populations classified as resistant to a discriminating herbicide dose. From 2017 to 2020, cleavers populations were screened with tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl. The percentages of tribenuron-methyl-resistant cleavers populations from 2017 to 2020 were 53.33%, 51.52%, 52.38% and 47.17%, respectively; and the percentages of cleavers populations with low tribenuron-methyl resistance were 23.33%, 26.67%, 30.00% and 36.67%, respectively. The percentages of bensulfuron-methyl-resistant cleavers populations from 2017 to 2020 were 36.67%, 39.39%, 35.71% and 33.96%, respectively; and the percentages of cleavers populations had low tribenuron-methyl resistance were 30.00%, 40.00%, 53.33% and 23.33%, respectively. The percentages of halosulfuron-methyl-resistant cleavers populations from 2017 to 2020 were 26.67%, 27.27%, 50.00% and 41.51%, respectively; and the percentages of cleavers population with low tribenuron-methyl resistance were 50.00%, 53.33%, 33.33% and 40.00%, respectively. Finally, 26.67%, 22.22%, 19.05% and 20.75% of cleavers populations had resistance to 2-methyl-4-chlorophenoxyacetic acid sodium (MCPA-Na) from 2017 to 2020, respectively; however, all populations were sensitive to fluroxypyr and carfentrazone-ethyl. This confirmation of multiple resistance in cleavers populations emphasizes the importance of diversity in herbicide sites of action as critical to extending the usefulness of remaining effective herbicides such as MCPA-Na, fluroxypyr and carfentrazone-ethyl for the management of this weed.

Keywords: *Galium aparine* L.; herbicide resistance; frequency; acetolactate synthase (ALS) inhibitor

1. Introduction

Cleavers (*Galium aparine* L.) is an annual broadleaved weed, which is widely distributed in major winter wheat (*Triticum aestivum* L.) farming fields in China [1]. Cleavers is an indigenous plant with rapid seedling development [2–4]; early flower initiation; creeping growth form that can cover the canopy of wheat and bring down crops that grow upright [5–7]; sticky foliage and fruits that can aid in its dispersal [8]; and with a similar size of seeds to wheat [6]. These characteristics have made cleavers a frequent and problematic weed, which competes for nutrients, light and water in wheat production [6]. According to the survey, the mixed occurrence of cleavers and gramineous weeds was the main composition of the weed community in wheat fields in the middle and lower reaches of the Yangtze River, and the population dominance of cleavers was up to 14.06%, which

was relatively high in Hubei, Anhui and Jiangsu Provinces [1,9]. Cleavers could cause a loss of wheat yield of 30 to 60% if the prevention and control application is improper [10].

Acetolactate synthase (ALS) is a key enzyme in the biosynthesis of the essential branched-chain amino acids such as valine, leucine and isoleucine [11,12]. Inhibition of ALS will affect the synthesis of the branched-chain amino acids with subsequent plant death [11,13]. Reduction in plant growth, shortening of internodes, purplish foliage and shortening of lateral roots are the injury symptoms on sensitive plants caused by the ALS-inhibiting herbicides [14]. ALS-inhibiting herbicides are key herbicides that provide broad-spectrum post-weed control, widely used throughout the world [15,16]. ALS is the target site of a large number of herbicides across the dissimilar sulfonylurea, imidazolinone, triazolopyrimidine, pyrimidinyl-thiobenzoates and sulfonyl-aminocarbonyl-triazolinone herbicide chemistries [17]. There are 54 registered ALS inhibitor herbicides globally, more than double the number of any other group, and they account for more than one-sixth of all registered herbicides [18]. ALS inhibitor herbicides have been used for over 40 years in all major crops and on most weed species with major differences in their selectivity, spectrum of control and residual activity [19–21].

Tribenuron-methyl is a principal kind of sulfonylurea (SU) herbicide that belongs to ALS-inhibiting herbicides, which was developed by Dupont. Tribenuron-methyl was registered in China in 1988 as a selective internal absorption conductive herbicide, and it has been widely used to control broad-leaved weeds in wheat fields in China since registration [13,14]. Tribenuron-methyl has been used in winter fields in China since registration, and its continuous use has led to the weakening of its control effect, especially in Jiangsu in recent years [22]. Bensulfuron-methyl, halosulfuron-methyl, MCPA-Na, carfentrazone-ethyl and fluroxypyr are the alternative or substitutive herbicides of tribenuron-methyl to control broad-leaf weeds in wheat fields, especially cleavers. Bensulfuron-methyl and halosulfuron-methyl belong to ALS-inhibiting sulfonylurea (SU) herbicides, the same as tribenuron-methyl. MCPA-Na and fluroxypyr are auxins herbicides, and carfentrazone-ethyl is protoporphyrinogen oxidase-inhibited herbicide. All these herbicides have been widely used in wheat production in China.

The first weed species resistant to ALS inhibitors, *Lactuca serriola* L., was identified in 1987 [23]. Following the persistent and extensive use of ALS inhibitors, 170 weed species have been documented as resistant around the world [24]. A total of 17 weed species have been documented as resistant to ALS inhibitors in China [24]. In 2008, Peng et al. found that the biotypes of cleavers, which were collected from winter wheat fields in Henan, Anhui and Shanxi provinces, were resistant to tribenuron-methyl [25]. Sun et al. (2011) reported that the nucleotide sequence of the R-biotype of cleavers differed from that of the S biotype with three amino acid substitutions, of which the amino acid substitution of Trp-574-Gly was located in the highly conserved region Domain B [26]. In 2016, Cui et al. found that 19 ALS-resistant mutant cleavers populations were detected from Henan, Anhui and Jiangsu provinces among 25 populations collected from five provinces. There were two mutant sites in total and three mutant patterns detected, which were Pro-197-Ser, Pro-197-ala and Trp-574-leu27. In 2016, the median effective dose (ED₅₀) values for tribenuron-methyl among the 12 cleavers populations that were obviously resistant to this herbicide were higher than its field-recommended dose among 20 populations from different regions of Jiangsu province [27,28].

At present, tribenuron-methyl is still one of the primary herbicides in Jiangsu Province to control cleavers in winter wheat fields, and it is still used repeatedly in large quantities. The herbicide exerts strong selection pressure because of its high activity on sensitive biotypes at the rates used and because of its soil residual activity [19]. Is there a new spread of cleavers resistance in Jiangsu Province? What is the change in the sensitivity of the cleavers population in Jiangsu Province to other dominant herbicides except for tribenuron-methyl? The answers to these questions are not clear.

In order to reveal the dynamics of the resistance of cleavers to major herbicides and develop prevention strategies in Jiangsu Province, we conducted a 4-year survey from

2017 to 2020 to evaluate the frequency and distribution of herbicide-resistant cleavers populations in Jiangsu. The objectives of this study were to (1) evaluate the frequency and distribution of tribenuron-methyl-, bensulfuron-methyl- and halosulfuron-methyl-resistant cleavers in Jiangsu, (2) evaluate multiple resistance patterns between six herbicides within cleavers populations, and (3) evaluate changes in the frequency and distribution of herbicide-resistant, cross-resistant and multiple-resistant cleavers over a 4-year time frame.

2. Materials and Methods

2.1. Seed Collection

Cleavers seeds were collected from wheat fields, fallow ground or scenic areas across Jiangsu Province in summer (May and June) each year from 2017 to 2020. In 2017, 30 samples were collected in the province, including 4 samples in southern Jiangsu, 15 samples in central Jiangsu and 11 samples in northern Jiangsu; in 2018, 36 samples were collected in the province, including 7 samples in Southern Jiangsu, 17 samples in central Jiangsu and 12 samples in northern Jiangsu; in 2019, 42 samples were collected in the province, including 8 samples in southern Jiangsu, 20 samples in central Jiangsu and 14 samples in northern Jiangsu; in 2020, 53 samples were collected in the province, including 18 samples in southern Jiangsu, 28 samples in central Jiangsu and 7 samples in northern Jiangsu. These collections were conducted by driving to all 13 cities around the province while maintaining only one sample per village to keep a minimum distance. The sampling locations were designed to separate cleavers collection sites while providing representative cleavers populations in production fields across the province without biasing the collection toward grower-reported cleavers control failures [29]. The cleavers seeds collected from the crop area were harvested from individuals that survived throughout the growing season, which could bias populations collected in these areas toward having an increased probability of herbicide resistance. Most sampling efforts were targeted at the central Jiangsu region, which is the main wheat production area in the province. A total of 159 populations were collected from wheat (*T. aestivum* (L.)) cropping systems, including one collection in the fallow ground and one collection on the field bank. Two cleavers populations were also collected from the scenic area. After a collection location had been selected, seeds were harvested from 20 to 30 individual cleavers plants to create a composite sample for that location (referred to as the population) [30]. The longitude and latitude were recorded for each location, and each location was geo-referenced using a handheld Global Positioning System (GPS) unit (A8, ZL Electronic Technology, Shanghai, China). Seeds were marked as collection sites, and temporarily kept in kraft paper bags in the field. After the seeds were brought back to the laboratory, they were dried naturally and stored at room temperature in the seed storage cabinet.

2.2. Greenhouse Screening

Cleavers seeds were cleaned with a combination of sieves and an air-blower prior to thorough blending to assure an even probability of screening progeny from each mature plant collected per location.

Horse liver soil (sandy loam soil) was used as the test soil. The pH value was 6.7, and the content of organic matter was 1.6%. After air drying and screening, the fine test soil was filled into 7 cm by 7 cm by 9 cm white plastic basin pots (with holes at the bottom). The pots were placed in plastic turnover boxes, and water was added to fill the bottom of the boxes to saturate the soil. The cleavers were soaked in cold distilled water at 4 °C for 7 days to break dormancy before seeding. A total of 18 seeds were sown in each pot, and covered with fine soil (about 0.5 cm thickness). The weeds were grown and watered daily in a greenhouse that had a 12-h/12-h photoperiod natural light with temperatures maintained between 20 ± 5 °C and 15 ± 5 °C. After the weeds emerged neatly, the seedlings were fixed to 12 plants per pot.

2.3. Herbicide Application and Cross-Resistance Bioassay

From 2017 to 2020, all cleavers populations (30 in 2017; 36 in 2018; 42 in 2019; and 53 in 2020) were screened with tribenuron-methyl, bensulfuron-methyl or halosulfuron-methyl, respectively.

For tribenuron-methyl treatments, 95% tribenuron-methyl technical was applied at 22.5 g a.i.hm⁻². For bensulfuron-methyl treatments, 96% bensulfuron-methyl technical (Institute of Ecomones, Changzhou, China) was applied at 75 g a.i.hm⁻². For halosulfuron-methyl treatments, 98% halosulfuron-methyl technical (Institute of Ecomones, Changzhou, China) was applied at 67.5 g a.i.hm⁻².

The treatment doses of herbicides were the upper limits of their recommended active ingredients in the field in China. The dose data was based on the registration records provided by the China Pesticide Information Network [31]. The herbicide technical was dissolved in acetone and diluted with 0.1% Tween-80 aqueous solution. Herbicide applications were sprayed by a bioassay spray tower (Nanjing Institute of Agricultural Mechanization, Nanjing, China), and the parameter of disc diameter was 50 cm; spindle rotation speed was 6 r·min⁻¹; nozzle aperture was 0.3 mm; the sprayer pressure was 0.3 MPa; droplet diameter was 100 µm; and nozzle flow was 90 mL·min⁻¹. The consumption of water was 450 L per hectare.

A blank control without herbicide application was set for every population, and four repetitions were set for each treatment.

Twenty-one days after treatment, the fresh weights of the weeds (the aboveground part) were measured, and the inhibition rates of fresh weight were calculated [32].

$$\text{Inhibition rate (\%)} = \frac{\text{Fresh weight (CK)} - \text{Fresh weight (treatment)}}{\text{Fresh weight (CK)}} \times 100$$

To describe the resistance level, we revised categories previously described by Owen et al. [33] and Westra et al. [30], classifying cleavers populations as either susceptible (inhibition rate ≥ 98%), having low resistance (inhibition rate between 80% and 98%) or resistant (inhibition rate ≤ 80%) to the respective discriminating herbicide rate for each herbicide.

2.4. Multiple Resistance

All cleavers populations were also screened separately with MCPA-Na, carfentrazone-ethyl and fluroxypyr, which are different types of herbicides to tribenuron-methyl. For MCPA-Na treatments, 88% MCPA-Na technical was applied at 1147.5 g a.i.hm⁻². For fluroxypyr treatments, 95% fluroxypyr technical (Institute of Ecomones, Changzhou, China) was applied at 210 g a.i.hm⁻². For carfentrazone-ethyl treatments, 95% carfentrazone-ethyl technical (Good Harvest, Nantong, China) was applied at 36 g a.i.hm⁻². MCPA-Na was dissolved and diluted with distilled water. Fluroxypyr was dissolved in acetone and diluted with 0.1% Tween-80 aqueous solution. Carfentrazone-ethyl was dissolved in toluene and diluted with 0.1% Tween-80 aqueous solution. The parameters of spray equipment, the procedures of herbicide application and the statistical methods were the same as those described in the previous paragraph.

2.5. Statistical Analysis

A randomized complete block design was used in all experiments with four replications, and each experiment was repeated three times. To determine whether significant difference of results was reached between experiment repeats, two-way ANOVA (SPSS 20.0 software, IBM Corporation, New York, NY, USA) was conducted for these results, where treatments were a fixed factor, experimental run (run) was a random factor and fresh weight inhibition rate was the dependent variable. The run and run by treatments interaction effects were nonsignificant for all the experiments. Thus, data were combined over the experiments for analysis [3].

3. Results

3.1. Tribenuron-Methyl Resistance and Cross-Resistance Survey in 2017

Thirty cleavers populations were screened for resistance to tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl to determine the frequency of resistance to evaluate the potential for and patterns of cross-resistance within and among cleavers populations. For tribenuron-methyl, 53.33% of populations had resistance, 23.33% of populations had low resistance and 23.33% were susceptible (Figure 1). For bensulfuron-methyl, 36.67% had resistance, 30.00% had low resistance and 33.33% were susceptible (Figure 1). For halosulfuron-methyl, 26.67% had resistance, 50.00% had low resistance and 23.33% were susceptible (Figure 1). Out of 30 populations, 76.67%, 66.67% and 76.67% had some level of resistance to tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl, respectively (Figures 1 and 2).

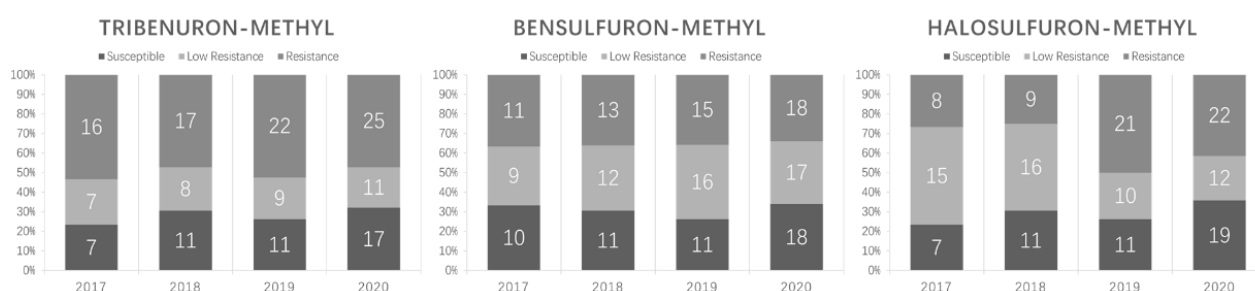


Figure 1. Distribution of cleavers seeds classified as susceptible (<2% survival), having low resistance (2% to 19% survival) and resistant (>20% survival) to a discriminating rate for tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl over the 4-year survey (2017 to 2020).

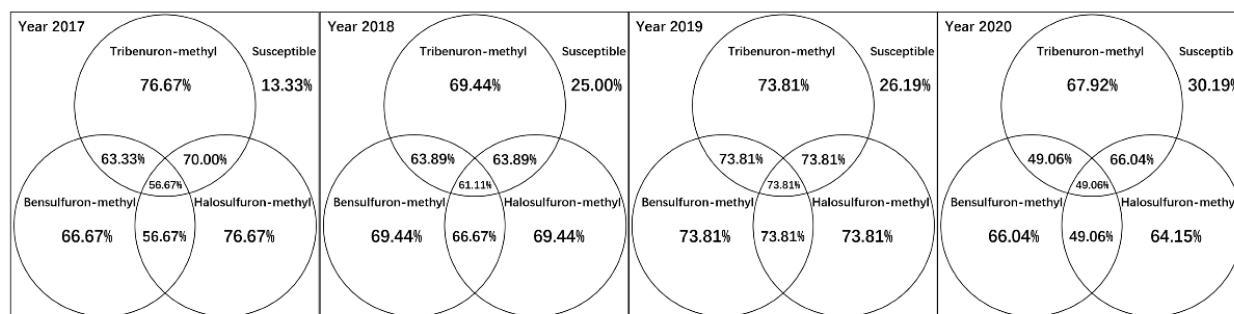


Figure 2. Proportion of cleavers seeds characterized as resistant (>20% survival) or having low resistance (2% to 19% survival) to three ALS-inhibiting sulfonylurea herbicides (tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl), and the proportion of seeds that were completely susceptible (<2% survival) to all three herbicides from 2017, 2018, 2019 and 2020.

For tribenuron-methyl and bensulfuron-methyl, 63.33% of populations had resistance and low resistance to both herbicides; 30.00% of populations had resistance to both herbicides; and 20.00% of populations were sensitive to both ingredients (Figure 2).

For tribenuron-methyl and halosulfuron-methyl, 70.00% of populations had resistance and low resistance to both herbicides; 23.33% of populations had resistance to both herbicides; and 16.67% of populations were sensitive to both ingredients (Figure 2).

For tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl, 56.67% of populations had resistance and low resistance to all herbicides; 16.67% of populations had resistance to all herbicides; and 13.33% of populations were sensitive to all the ingredients (Figure 2).

3.2. Tribenuron-Methyl Resistance and Cross-Resistance Survey in 2018

Thirty-six cleavers populations were screened for resistance to tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl. For tribenuron-methyl, 47.22% of populations had resistance, 22.22% of populations had low resistance and the remaining 30.56% were susceptible (Figure 1). For bensulfuron-methyl, 36.11% had resistance, 33.33% had low resistance and the remaining 30.56% were susceptible (Figure 1). For halosulfuron-methyl, 25.00% had resistance, 44.44% had low resistance and 30.56% were susceptible (Figure 1). Out of 36 populations, 69.44% had some level of resistance to all three tested herbicides, respectively (Figures 1 and 2).

For tribenuron-methyl and bensulfuron-methyl, 63.89% of populations had resistance and low resistance to both herbicides; 27.78% of populations had resistance to both herbicides; and 25.00% of populations were sensitive to both ingredients (Figure 2).

For tribenuron-methyl and halosulfuron-methyl, 63.89% of populations had resistance and low resistance to both herbicides; 22.22% of populations had resistance to both herbicides; and 25.00% of populations were sensitive to both ingredients (Figure 2).

For tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl, 61.11% of populations had resistance and low resistance to all herbicides; 16.67% of populations had resistance to all herbicides; and 25.00% of populations were sensitive to all the ingredients (Figure 2).

3.3. Tribenuron-Methyl Resistance and Cross-Resistance Survey in 2019

Forty-two cleavers populations were screened for resistance to tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl. For tribenuron-methyl, 52.38% of populations had resistance, 21.43% of populations had low resistance and the remaining 26.19% were susceptible (Figure 1). For bensulfuron-methyl, 35.71% had resistance, 38.10% had low resistance and the remaining 26.19% were susceptible (Figure 1). For halosulfuron-methyl, 50.00% had resistance, 23.81% had low resistance and the remaining 26.19% were susceptible (Figure 1). Out of 42 populations, 73.81% had some level of resistance to all three tested herbicides, respectively (Figures 1 and 2).

For tribenuron-methyl and bensulfuron-methyl, 73.81% of populations had resistance and low resistance to both herbicides; 35.71% of populations had resistance to both herbicides; and 26.19% of populations were sensitive to both ingredients (Figure 2).

For tribenuron-methyl and halosulfuron-methyl, 73.81% of populations had resistance and low resistance to both herbicides; 42.86% of populations had resistance to both herbicides; and 26.19% of populations were sensitive to both ingredients (Figure 2).

For tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl, 73.81% of populations had resistance and low resistance to all herbicides; 28.57% of populations had resistance to all herbicides; and 26.19% of populations were sensitive to all the ingredients (Figure 2).

3.4. Tribenuron-Methyl Resistance and Cross-Resistance Survey in 2020

Fifty-three cleavers populations were screened for resistance to tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl. For tribenuron-methyl, 47.17% of populations had resistance, 20.75% of populations had low resistance and 32.08% were susceptible (Figure 1). For bensulfuron-methyl, 33.96% had resistance, 32.08% had low resistance and 33.96% were susceptible (Figure 1). For halosulfuron-methyl, 41.51% had resistance, 22.64% had low resistance and 35.85% were susceptible (Figure 1). Out of 53 populations, 67.92%, 66.04% and 64.15% had some level of resistance to tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl, respectively (Figures 1 and 2).

For tribenuron-methyl and bensulfuron-methyl, 49.06% of populations had resistance and low resistance to both herbicides; 35.85% of populations had resistance to both herbicides; and 30.19% of populations were sensitive to both ingredients (Figure 2).

For tribenuron-methyl and halosulfuron-methyl, 66.04% of populations had resistance and low resistance to both herbicides; 35.85% of populations had resistance to both herbicides; and 30.19% of populations were sensitive to both ingredients (Figure 2).

For tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl, 49.06% of populations had resistance and low resistance to all herbicides; 35.85% of populations had resistance to all herbicides; and 30.19% of populations were sensitive to all the ingredients (Figure 2).

3.5. Multiple Resistance

All cleavers populations collected over a 4-year period were screened for the phenoxyalkanoic acids herbicide MCPA-Na, pyridines herbicide fluroxypyr and triazolinones herbicide carfentrazone-ethyl.

A total of 26.67%, 22.22%, 19.05% and 20.75% cleavers populations had resistance to MCPA-Na in the 4 years from 2017 to 2020, respectively; however all populations were sensitive to fluroxypyr and carfentrazone-ethyl (Figure 3).

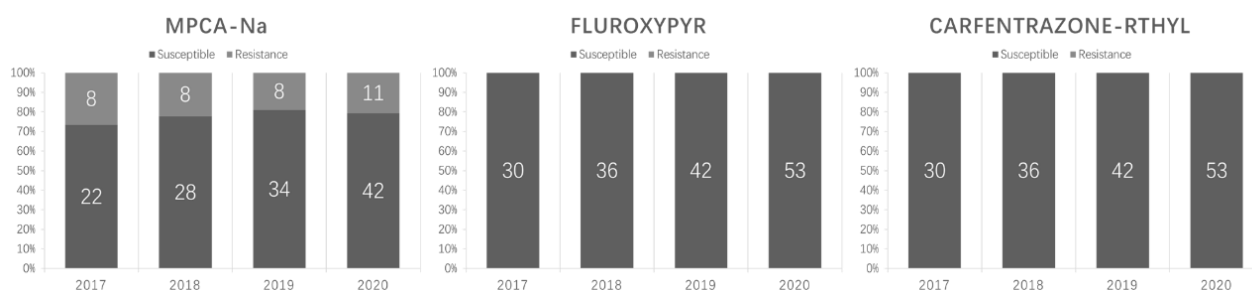


Figure 3. Distribution of cleavers seeds classified as susceptible (<2% survival), having low resistance (2% to 19% survival) and resistant (>20% survival) to a discriminating rate for MCPA-Na, fluroxypyr and carfentrazone-ethyl over the 4-year survey period (2017 to 2020).

For tribenuron-methyl and MCPA-Na, 23.33% of populations had resistance and 20.00% of populations were sensitive to both ingredients in 2017; 19.00% of populations had resistance and 28.00% of populations were sensitive to both ingredients in 2018; 19.05% of populations had resistance and 26.19% of populations were sensitive to both ingredients in 2019; and 16.98% of populations had resistance and 26.42% of populations were sensitive to both ingredients in 2020 (Figures 4 and 5).

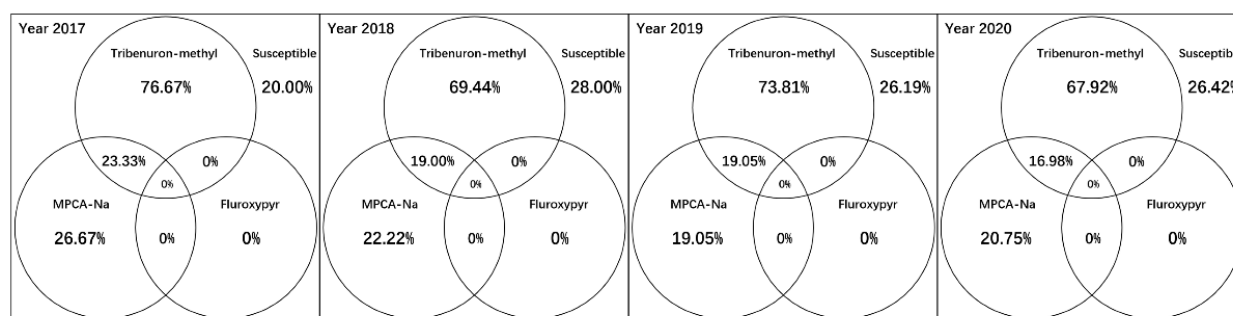


Figure 4. Proportion of cleavers seeds characterized as resistant (>20% survival) or having low resistance (2% to 19% survival) to tribenuron-methyl and two auxin herbicides (MPCA-Na and Fluroxypyr), and the proportion of seeds that were completely susceptible (<2% survival) to all three herbicides from 2017, 2018, 2019 and 2020.

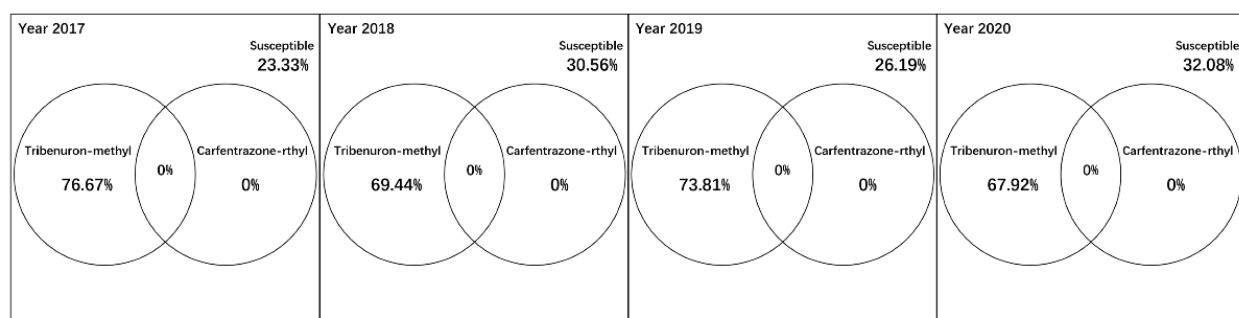


Figure 5. Proportion of cleavers seeds characterized as resistant (>20% survival) or having low resistance (2% to 19% survival) to tribenuron-methyl and a protoporphyrinogen oxidase inhibited herbicide (carfentrazone-rthyl), and the proportion of seeds that were completely susceptible (<2% survival) to both herbicides from 2017, 2018, 2019 and 2020.

4. Discussion

The proportion of the populations resistant to tribenuron-methyl of cleavers in Jiangsu was 76.67%, 69.44%, 73.81% and 67.92% from 2017 to 2020. The resistance frequency fluctuated slightly, although it remained between 67% and 77% over the four years. The proportion of resistant populations to tribenuron-methyl was high, and tribenuron-methyl has been unable to control the damage to cleavers effectively in most regions of Jiangsu. In terms of geographical distribution, 60% to 67% cleavers were resistant to tribenuron-methyl in southern Jiangsu; 75% to 89% in central Jiangsu; and 66% to 88% in northern Jiangsu.

The proportion of the populations resistant to bensulfuron-methyl and halosulfuron-methyl of cleavers in Jiangsu was 64% to 77% from 2017 to 2020. The data fluctuated slightly between years, and the resistance frequency was high. ALS inhibitors bensulfuron-methyl and halosulfuron-methyl were ineffective against tribenuron-methyl-resistant cleavers. The population was similar to that of tribenuron-methyl-resistant cleavers. The resistance showed as slightly light in southern Jiangsu, while the resistance in central and northern Jiangsu showed as strong.

The proportion of the populations resistant to MCPA-Na of cleavers was 19% to 26% in Jiangsu from 2017 to 2020. The data fluctuated slightly between years, and the resistance frequency was low. MCPA-Na could basically control the damage in tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl-resistant cleavers. There was no significant difference in the distribution of MCPA-Na resistant cleavers in different regions. All collected cleavers populations were sensitive to fluroxypyr and carfentrazone-ethyl from 2017 to 2020, and there was no difference between different years.

As some relatively high resistance risk herbicides, the long-term use of ALS inhibitor easily leads to the mutation or the increasing expression of ALS amino acid sequence, which eventually cause herbicide resistance. This study found that more than 67% of cleavers populations were resistant to tribenuron-methyl in Jiangsu Province. The proportions of bensulfuron-methyl- and halosulfuron-methyl-resistant cleavers were more than 64%, which were similar to the frequency of tribenuron-methyl cleavers. However, all collected cleavers populations showed no resistance to fluroxypyr and carfentrazone-ethyl, which indicated that the mutation of the target enzyme ALS gene and the increase in gene expression may be the main factor in the resistance. Several studies have discovered that the amino acid substitution of ALS was the main reason for the resistance of weeds [12,22,26,34–41]. Further research about specific mechanism is needed.

The high frequency of ALS inhibitors-resistant cleavers creates a huge threat to the sustainability of ALS inhibitors usage. In this study, all populations were sensitive to fluroxypyr and carfentrazone-ethyl. Fluroxypyr and carfentrazone-ethyl could be used to control cleavers in Jiangsu province, but it will result in accumulation of resistance mechanisms due to the high selection pressure if these herbicides are used continuously over the years [15]. Introducing a new site of herbicide action may be a useful strategy

to manage resistant cleavers; however, the evolution of herbicide-resistant weeds has in fact outpaced the development of new herbicidal active ingredients in the past two decades [42,43]. The importance of integrated weed management approaches, maximizing diversity in cropping systems, depleting the soil seedbank, avoiding the introduction of new weed seeds and investing more in prevention should be emphasized in the control of resistant cleavers.

Several studies have found that ALS gene mutation or gene overexpression can lead to the resistance of weeds to ALS inhibitor herbicides, while the enhanced metabolic capacity mediated by cytochrome p450 oxidases can lead to the resistance of weeds to ALS inhibitor herbicides and herbicides with other action mechanisms [44–49]. We found that the population of cleavers in different regions of Jiangsu Province had obvious resistance to the ALS inhibitors such as tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl, but was sensitive to the auxin herbicides and PPO inhibitors. The resistance of cleavers in different regions of Jiangsu Province may be caused by the mutation or overexpression of its ALS gene, but the specific resistance mechanism needs to be further studied.

5. Conclusions

The proportion of the cleavers populations resistant to tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl in Jiangsu was between about 64% and 77% from 2017 to 2020. The data fluctuated slightly between years, and the resistance frequency was high. Tribenuron-methyl, bensulfuron-methyl and halosulfuron-methyl were not recommended for controlling cleavers in Jiangsu Province in recent years. Meanwhile, the proportion of cleavers populations resistant to 2-methyl-4-chlorophenoxyacetic acid sodium (MCPA-Na) was between 19% and 27%. MCPA-Na could be used to control cleavers; however long-term continuous use is not recommended. In contrast to the above, all collected cleavers seeds were susceptible to fluroxypyr and carfentrazone-ethyl. As the recommended herbicides, both ingredients could control the tribenuron-methyl-, bensulfuron-methyl- and halosulfuron-methyl-resistant cleavers effectively in Jiangsu Province.

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