Field Resistance of *Digitaria sanguinalis* (L.) Scop. to Haloxyfop-P-methyl in China’s Cotton Fields

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Abstract: Large crabgrass, *Digitaria sanguinalis* (L.) Scop., is a devastating weed species in the cotton (*Gossypium* spp.) fields in China. It has developed resistance to haloxyfop-P-methyl, an aryloxyphenoxypropionate herbicide known for its ability to inhibit lipid synthesis and induce oxidative stress in weeds, due to years of continuous and intensive use. Here, we present the results from a nation-wide, long-term resistance monitoring effort. To understand the scale and level of haloxyfop-P-methyl resistance, a total of 65 *D. sanguinalis* populations from eight cotton production provinces, including Hunan, Jiangxi, Xinjiang, Henan, Hubei, Hebei, Shanxi, and Anhui, were collected from 2014–2017. Based on results from dose response to haloxyfop-P-methyl, we observed a gradient of sensitivity to haloxyfop-P-methyl among 65 field populations, ranging from sensitive (8), to low-level resistance (40; 2 ≤ RI ≤ 10) to moderate-level resistance (17; 10 < RI < 20). Although no high-level resistance (RI > 20) was found among the 65 populations, populations from Hunan and Hebei exhibited a rapid spread of field-evolved resistance. After challenged with haloxyfop-P-methyl (48.600 g a.i./ha at the 4–5-leaf stage), resistant and susceptible *D. sanguinalis* responded differently in the activity of an array of resistance-related enzymes, including acetyl-CoA carboxylase (ACCase), glutathione S-transferase (GSTs), nicotinamide-adenine dinucleotide phosphate (NADPH) and carboxylesterase (CarE), suggesting the potential involvement of NADPH, CarE and GSTs in *D. sanguinalis* to haloxyfop-P-methyl resistance.

Keywords: herbicide resistance; *Digitaria sanguinalis*; haloxyfop-P-methyl; acetyl-CoA carboxylase; glutathione S-transferase

1. Introduction

By competing with crop plants for nutrients, water, light and space, agricultural weeds have always been a major cause of crop loss. The use of herbicides is often considered the most effective way of controlling weeds. However, weed species can evolve resistance to herbicides under persistent and intensive herbicide selection. To date, there are 512 unique cases of herbicide-resistant weeds globally, with 266 weeds species having evolved resistance to 165 different herbicides. Herbicide-resistant weeds have been reported in 96 crops in 71 countries [1].

Cotton (*Gossypium* spp.) is a highly important economic crop in China. Both the largest cotton production industry and consumer market are in China [2]. However, weeds are one of the most significant constraints to China’s cotton production [3]. Large crabgrass (*Digitaria sanguinalis* (L.) Scop.), an annual graminaceous weed with a high reproductive capability by which almost all cut nodes with fibrous roots and buds can develop into new plants, is one of the most problematic weed species in cotton fields in China [4,5]. Because of the climatic adaptability, large crabgrass can grow and reproduce in most areas, such as the Tibet Autonomous Region, Xinjiang Uygur Autonomous Region, Jiangsu Province,
Shandong Province, Hunan Province, Sichuan Province, Jiangxi Province, Shaanxi Province, Gansu Province, Shanxi Province and Hebei Province [6]. These areas cover the main cotton growing regions in China.

Weed resistance to herbicides is defined as the inheritable ability of individual plants to survive after repeated use of a compound that controls the original population [7,8]. Since 1985, acetyl-CoA carboxylase (ACCase)-inhibiting herbicides have been widely used to control graminaceous weeds, including large crabgrass [9]. Selection by ACCase-inhibiting herbicides could rapidly result in weed resistance after three to five years of constant use [10]. Currently, there are 48 species of graminaceous weeds resistant to ACCase-inhibiting herbicides, including *Alopecurus myosuroides*, resistant to haloxyfop-methyl and fenoxaprop-ethyl; *Beckmannia syzigachne*, resistant to fenoxaprop-ethyl; *Polypogon fugax*, resistant to fluazifop-butyl, quizalofop-ethyl and fenoxaprop-ethyl; and *Echinochloa colona*, resistant to fenoxaprop-ethyl [1]. It was reported that *D. sanguinalis* was resistant to fluazifop-P-butyl, haloxyfop-ethoxyethyl and quizalofop-P-ethyl in onions (*Allium cepa*) and vegetables [11,12]. Meanwhile, the resistance mechanisms of ACCase inhibitors are generally target-site-based resistance (TSR) [13]. Studies have shown that most of the cases are caused by an alteration of the target site, and mutations in the CT domain have been identified, including at amino acids 1781 [14,15], 1999 [16], 2027 [17], 2041 [15], 2078 [14], 2088 [18] and 2096 [15,17]. Recent work also showed constitutive overexpression and duplication of the herbicide target gene of *D. sanguinalis* conferring resistance to fluazifop-P-butyl and possibly to other ACCase-inhibiting herbicides [19].

In addition to TSR, non-target-site-based resistance (NTSR) can also confer resistance to ACCase inhibitors. NTSR includes all resistance mechanisms, with no alteration to the target site [20]. Researchers know little about them compared with TSR in graminaceous weeds. These NTSR mechanisms typically focus on reduced translocation, enhanced metabolism and decreased rate of herbicide activation and sequestration [21]. Of the NTSR mechanisms, enhanced herbicide metabolism is considered the mechanism of resistance to ACCase-inhibiting herbicides [22,23]. Metabolic resistance generally manifests a low level of resistance; however, it can reduce herbicide effects in herbicides with similar or different modes of action [24,25]. Among the metabolic resistance, P450s and glutathione S-transferase (GSTs) could play important roles in conferring NTSR to weed species. Composing a heterogeneous group of enzymes, GSTs possess the ability to detoxify herbicides [25]. Plant GSTs have also been identified as functioning in primary and secondary metabolism; herbicide detoxification; and plant protection against oxidative damage, heavy metals and xenobiotic compartment [26–28]. Nicotinamide-adenine dinucleotide phosphate (NADPH) is produced in plant photosynthesis by the transduction of sunlight energy for the carbon reduction cycle. Only by using electrons from NADPH can P450s catalyze the activation of molecular oxygen in herbicide metabolism [29,30]. Indeed, NADPH oxidase was identified as an enzymatic source of electrons that trigger paraquat redox cycling by microglia [31]. As a multifunctional protein, glutathione S-transferase (GSTs) is involved in a variety of intracellular functions, including catalyzing the nucleophilic attack of the tripeptide glutathione (GSH) [32]. Therefore, understanding both P450s and GSTs in plants seems to be highly important for controlling metabolic-resistant weeds.

Haloxyfop-P-methyl, an ACCase-inhibiting herbicide, targets the carboxylase-transferase (CT) domain of the plastidic ACCase and can be lethal to plants because of their ability to inhibit fatty acid biosynthesis by preventing the adenosine triphosphate (ATP)-dependent carboxylation of acetyl-CoA from catalyzing to malonyl-CoA [33–36]. During the xenobiotic biotransformation, plant carboxylesterase (CarE) can activate haloxyfop-P-methyl to be formulated as esters to ease cuticle penetration, but haloxyfop esters are rapidly hydrolyzed to haloxyfop acid, which are more phytotoxic than the ester forms [37,38]. Thus, plant carboxylesterase has a crucial importance in haloxyfop-P-methyl biotransformation in plants.

Glyphosate, quizalofop-P-ethyl and haloxyfop-P-methyl are commonly used for *D. sanguinalis* control in China. Researchers in China had reported that *Alopecurus aequalis* Sobol. in rape fields and *Eleusine indica* in cotton fields manifest haloxyfop-P-methyl
resistance [39,40]. Based on our field resistance detection, the efficacy of haloxyfop-P-methyl on D. sanguinalis has also greatly decreased in cotton fields. However, there are few investigations studying the resistance level of D. sanguinalis to haloxyfop-P-methyl in China’s cotton fields. Regarding the resistance of D. sanguinalis to other herbicide groups, studies only confirmed that D. sanguinalis was resistant to imazethapyr, nicosulfuron and flumetsulam in maize fields in China [5,41]. Therefore, it is necessary to identify resistance levels and understand the possible reason for the D. sanguinalis resistance to haloxyfop-P-methyl. Here, the purpose of this paper was to (1) determine the resistance levels to haloxyfop-P-methyl of different D. sanguinalis populations in China, and (2) identify the activity of ACCase, GSTs, P450s and CarE in D. sanguinalis that might be related to the resistance to haloxyfop-P-methyl.

2. Materials and Methods

2.1. Plant Materials

During September and October of 2014 to 2017, seeds from a total of 65 populations of D. sanguinalis were harvested randomly from the main cotton production regions in China, including: Hunan (HN), Jiangxi (JX), Xinjiang (XJ), Henan (HEN), Hubei (HB), Hebei (HEB), Shanxi (SX) and Anhui (AH) Provinces (Supplementary Tables S1–S4). Mature seeds of the susceptible (S, 14HN1) population were harvested from a remote area that had never been treated with ACCase-inhibiting herbicides in Li County in Hunan Province. We selected the plump seeds after they were air-dried at room temperature for 7 d, and then stored them in sealed glass bottles at 20 °C and 30% RH in the dark for six months.

Seeds of D. sanguinalis were first immersed in distilled water for 24 h and then germinated in Petri dishes for 36 h. The germinated seedlings were transplanted into 9 cm diameter plastic pots that contained moist loam soil, which were then maintained in a climate chamber at 30 °C/25 °C (light/dark). All of the above-mentioned seedlings were exposed to a 14 h light period with a light intensity of 12,500 lux. The D. sanguinalis seedlings were subsequently thinned down to 12 per plastic pot before herbicide application.

2.2. Dose Response to Haloxyfop-P-methyl

Whole-plant dose–response experiments were conducted to determine the GR50 (herbicide application dose causing a 50% growth reduction in plants relative to that of the controls) of haloxyfop-P-methyl (commercial haloxyfop-P-methyl 108 g/L emulsifiable concentrate was from Dow Chemical Company, Midland, Michigan, United States). Herbicide was applied to the seedlings at the 4–5-leaf stage. The S seedlings were treated with haloxyfop-P-methyl at rates of 0.759, 1.519, 3.038, 6.075, 12.150, 24.300 g a.i./ha using a moving-boom cabinet sprayer delivering 600 L/ha water at a pressure of 0.4 MPa by a flat fan nozzle positioned 30 cm above the foliage, while others were treated at 1.519, 3.038, 6.075, 12.150, 24.300, 48.600 g a.i./ha. After haloxyfop-P-methyl treatment, the plants were returned to the climate chamber and watered every other day. All of the aboveground biomass for each haloxyfop-P-methyl treatment was harvested after 14 d and dried at 60 °C for 72 h; the dry weights were subsequently recorded. The experiment was conducted with three replications per herbicide dose.

Resistance index (RI) was calculated by the GR50 of the field population divided by that of the S population to estimate the different resistance levels in this paper, including low-level resistance (2 ≤ RI ≤ 10), moderate-level resistance (10 < RI < 20) and high-level resistance (RI > 20).

2.3. ACCase Activity Assays

ACCase activity was analyzed in this study in August 2016. Two populations (15HN10, 15HN1) were randomly chosen from the bioassay results with a moderate RI to compare with the S population (14HN1) in terms of enzyme activities.

Seedlings from the R (15HN10, 15HN1) and S (14HN1) populations were grown in a plant growth chamber and treated with haloxyfop-P-methyl at 48.600 g a.i./ha at
the 4–5-leaf stage. One gram of leaf tissue from the R and S plants was harvested for extraction of ACCase at 1, 2, 3, 4, 6, 8 and 10 d after haloxyfop-P-methyl spraying, while tissues from the untreated plants of each population were also harvested as a control. ACCase was partially purified at 4 °C according to the procedures of an ACCase kit (Comin Biotechnology Company, Suzhou, China). All chemicals used were from the Comin kit unless otherwise specified. Following the instructions of the kit, ACCase activity was determined by measuring the increase in inorganic phosphorus by the ammonium molybdate method. The amount of 1 µmol inorganic phosphorus produced per milligram of protein per hour was one ACCase activity unit (U). Each experimental unit was one enzyme assay. The protein contents of the extracts were determined according to the Bradford method [42]. The experiment was completely randomized with three replicates per treatment.

2.4. GST Activity Assays

GST activity assays were performed as previously described according to the procedures of a GST kit (Comin Biotechnology Company, Suzhou, China) in August 2016. One gram of leaf tissue from the R (15HN10, 15HN1) and S (14HN1) plants was harvested for extraction of GSTs at 1, 2, 3, 4, 5, 6, 7 and 8 d after haloxyfop-P-methyl spraying, while tissues from the untreated plants of each population were also harvested as a control. The GST activity was determined by following the instructions of the kit. GSTs catalyzed the combination of GSH and CDNB, and the light absorption peak wavelength of the combined product was 340 nm. The GST activity was calculated by measuring the increase rate of absorbance at the wavelength of 340 nm. GST activity (U): 1 µmol/L CDNB binds to GSH per mg protein per minute at 25 °C. The experiment was completely randomized with three replicates per treatment.

2.5. NADPH Activity Assays

NADPH activity assays were also performed as previously described according to the procedures of an NADPH kit (Comin Biotechnology Company, Suzhou, China) in August 2016. One gram of leaf tissue from the R (15HN10, 15HN1) and S (14HN1) plants was harvested for extraction of NADPH at 1, 2, 3, 4, 6, 8 and 10 d after haloxyfop-P-methyl spraying, while tissues from the untreated plants of each population were also harvested as a control. NADPH activity was determined by following the instructions of the kit. NADPH catalyzed oxidized cytochrome c to produce reduced cytochrome c, which had a characteristic absorption peak at 550 nm. NADPH activity was calculated by measuring the increased rate of reduced cytochrome c. An amount of 1 nmol of reduced cytochrome c was catalyzed to produce 1 nmol of reduced cytochrome c per mg of protein per minute as 1 unit of NADPH activity. The experiment was completely randomized with three replicates per treatment.

2.6. CarE Activity Assays

CarE activity assays were also performed as described above according to the procedures of a CarE kit (Comin Biotechnology Company, Suzhou, China) in August 2016. One gram of leaf tissue from the R (15HN10, 15HN1) and S (14HN1) plants was harvested for extraction of CarE at 1, 2, 3, 4, 6 and 8 d after haloxyfop-P-methyl spraying, while tissues from the untreated plants of each population were also harvested as a control. CarE activity was determined by following the instructions of the kit. CarE could catalyze the formation of naphthyl acetate from 1-naphthyl acetate, and fast blue color developed; CarE activity was calculated by the increased rate of light absorption at 450 nm. The catalytic absorbance value of each mg protein increased by 1 per minute in the reaction system at 37 °C, which was defined as one CarE activity unit. The experiment was completely randomized, with three replicates per treatment.
3. Statistical Analysis

Analysis of the whole-plant dose–response data was calculated using the following probit model of SPSS software (Version 19.0, IBM SPSS Statistics, New York, NY, USA). The data of ACCase, GSTs, NADPH and CarE activity assays were analyzed through the software SigmaPlot (Version 10.0, Systat Software Inc., San Jose, CA, USA). Data of three replications were averaged for calculation. All the data were subjected to analysis of variance, and standard deviation values were calculated.

Enzyme relative activity (GSTs, NADPH, CarE) was calculated using Equation (1), where \( E \) is relative activity, \( E_n \) is the enzyme activity on day \( n \) after application, and \( E_0 \) is the enzyme activity before application:

\[
E = \frac{E_n}{E_0}
\]  

(1)

Haloxyfop-P-methyl application dose causing 50% a growth reduction in plants in dry weight relative to that of the controls (GR_{50}) was computed by probit regression model:

\[
Y = A + BX
\]  

(2)

where \( Y \) is probit growth corresponding to dry weight expressed as a percentage of the untreated control, \( A \) is intercept, \( B \) is slope and \( X \) is \( \log_{10}(\text{dose}) \).

4. Results

4.1. Dose Response to Haloxyfop-P-methyl

Results showed that 8 of 65 populations were sensitive to haloxyfop-P-methyl, and low-level resistance (2 ≤ RI ≤ 10) to haloxyfop-P-methyl was found in 40 populations, while 17 populations exhibited a moderate resistance level (10 < RI < 20). Among the 13 Hunan populations in 2013, the average resistance index was only 3.88, of which 14HN9 had the highest GR_{50} 16.430 g a.i./ha. By 2014, the average resistance index of nine Hunan populations rose to 7.91, of which 15HN1 had the highest GR_{50}, reaching 28.089 g a.i./ha.

In the 65 populations, no high-level resistance (RI > 20) was found (Tables 1–4). However, the results confirmed that resistance of *D. sanguinalis* to haloxyfop-P-methyl in China’s cotton fields was increasing year by year.

| Table 1. The different resistance levels of *D. sanguinalis* populations to haloxyfop-P-methyl in cotton fields (2014). |
|---------------------------------------------------------------|
| Population | Correlation Coefficient | GR_{50} (g a.i./hm^2) | Resistance Index |
|------------|-------------------------|------------------------|-----------------|
| 14HN2      | 0.9701                  | 5.722                  | 3.115           |
| 14HN3      | 0.9059                  | 6.648                  | 3.619           |
| 14HN4      | 0.9584                  | 3.189                  | 1.736           |
| 14HN5      | 0.9571                  | 3.350                  | 1.824           |
| 14HN6      | 0.9906                  | 1.921                  | 1.046           |
| 14HN7      | 0.9601                  | 2.114                  | 1.151           |
| 14HN8      | 0.9075                  | 2.824                  | 1.537           |
| 14HN9      | 0.9544                  | 16.430                 | 8.944           |
| 14HN10     | 0.9500                  | 18.150                 | 9.880           |
| 14HN11     | 0.9822                  | 9.446                  | 5.142           |
| 14HN12     | 0.9850                  | 15.098                 | 8.219           |
| 14HN13     | 0.9888                  | 5.801                  | 3.158           |
| 14JX1      | 0.9516                  | 11.519                 | 6.271           |
| 14HEN1     | 0.9529                  | 7.575                  | 4.124           |
| 14HEN2     | 0.9736                  | 11.038                 | 6.009           |
| 14HB1      | 0.9823                  | 4.483                  | 2.440           |
| 14HB2      | 0.9688                  | 22.845                 | 12.436          |
| 14SX1      | 0.9903                  | 9.299                  | 5.062           |
| 14JX1      | 0.9755                  | 21.783                 | 11.858          |
Table 1. Cont.

| Population | Correlation Coefficient | GR50 (g a.i./hm²) | Resistance Index |
|------------|--------------------------|-------------------|------------------|
| 13HEB1     | 0.9669                   | 18.751            | 10.207           |
| 13HEB2     | 0.9753                   | 16.001            | 8.710            |
| 13AH1      | 0.9606                   | 19.492            | 10.611           |
| 13HN1(S)   | 0.9925                   | 1.837             | 1.000            |

GR50, herbicide application dose causing a 50% growth reduction in plants in terms of dry weight relative to that of the controls; resistance index (RI) = the GR50 values of different D. sanguinalis populations/that of the S population.

Table 2. The different resistance levels of D. sanguinalis populations to haloxyfop-P-methyl in cotton fields (2015).

| Population | Correlation Coefficient | GR50 (g a.i./hm²) | Resistance Index |
|------------|--------------------------|-------------------|------------------|
| 15HN1      | 0.9729                   | 28.089            | 16.770           |
| 15HN2      | 0.9530                   | 15.032            | 8.974            |
| 15HN3      | 0.9549                   | 6.384             | 3.811            |
| 15HN5      | 0.9650                   | 4.909             | 2.931            |
| 15HN6      | 0.9550                   | 14.557            | 8.691            |
| 15HN8      | 0.9512                   | 8.067             | 4.816            |
| 15HN9      | 0.9523                   | 10.897            | 6.506            |
| 15HN10     | 0.9502                   | 16.834            | 10.050           |
| 15HN12     | 0.9534                   | 14.439            | 8.620            |
| 15HEN1     | 0.9693                   | 4.770             | 2.848            |
| 15HEN2     | 0.9821                   | 3.788             | 2.261            |
| 15HX1      | 0.9775                   | 3.977             | 2.374            |
| 15HX2      | 0.9701                   | 3.346             | 1.998            |
| 15HX3      | 0.9665                   | 6.529             | 3.898            |
| 15HX4      | 0.9590                   | 4.268             | 2.548            |
| 15HX7      | 0.9948                   | 2.656             | 1.586            |
| 15HB1      | 0.9596                   | 17.407            | 10.392           |
| 15HEB2     | 0.9501                   | 3.681             | 2.198            |
| 14HN1(S)   | 0.9586                   | 1.675             | 1.000            |

GR50, herbicide application dose causing a 50% growth reduction in plants in terms of dry weight relative to that of the controls; resistance index (RI) = the GR50 values of different D. sanguinalis populations/that of the S population.

Table 3. The different resistance levels of D. sanguinalis populations to haloxyfop-P-methyl in cotton fields (2016).

| Population | Correlation Coefficient | GR50 (g a.i./hm²) | Resistance Index |
|------------|--------------------------|-------------------|------------------|
| 16HN2      | 0.9867                   | 8.004             | 4.717            |
| 16HN3      | 0.9776                   | 6.711             | 3.955            |
| 16HN4      | 0.9764                   | 4.535             | 2.673            |
| 16HN5      | 0.9917                   | 5.892             | 3.472            |
| 16JX1      | 0.9168                   | 11.558            | 6.811            |
| 16JX4      | 0.9801                   | 5.258             | 3.099            |
| 16HEN1     | 0.9559                   | 4.614             | 2.791            |
| 16HEB1     | 0.9777                   | 5.051             | 2.976            |
| 16HEB4     | 0.9837                   | 8.607             | 5.072            |
| 16HEB5     | 0.9939                   | 6.757             | 3.982            |
| 16HEB6     | 0.9896                   | 5.788             | 3.411            |
| 16HEB7     | 0.9884                   | 3.938             | 2.320            |
| 14HN1(S)   | 0.9819                   | 1.697             | 1.000            |

GR50, herbicide application dose causing a 50% growth reduction in plants in terms of dry weight relative to that of the controls; resistance index (RI) = the GR50 values of different D. sanguinalis populations/that of the S population.
Table 4. The different resistance levels of *D. sanguinalis* populations to haloxyfop-P-methyl in cotton fields (2017).

| Population | Correlation Coefficient | GR$_{50}$ (g a.i./hm$^2$) | Resistance Index |
|------------|--------------------------|-----------------------------|-----------------|
| 17HEB1     | 0.9856                   | 22.888                      | 12.399          |
| 17HEB2     | 0.9911                   | 21.128                      | 11.446          |
| 17HEB3     | 0.9804                   | 22.991                      | 12.454          |
| 17HEB4     | 0.9865                   | 22.204                      | 12.028          |
| 17HEB5     | 0.9927                   | 22.670                      | 12.281          |
| 17HEB6     | 0.9922                   | 30.024                      | 16.264          |
| 17HEB7     | 0.9719                   | 21.777                      | 11.797          |
| 17HEB8     | 0.9960                   | 20.974                      | 11.362          |
| 17HEB9     | 0.9887                   | 10.502                      | 5.689           |
| 17JX1      | 0.9722                   | 7.998                       | 4.333           |
| 17JX2      | 0.9717                   | 14.148                      | 7.664           |
| 17JX3      | 0.9883                   | 21.739                      | 11.776          |
| 14HN1(S)   | 0.9920                   | 1.846                       | 1.000           |

GR$_{50}$, herbicide application dose causing a 50% growth reduction in plants in terms of dry weight relative to that of the controls; resistance index (RI) = the GR$_{50}$ values of different *D. sanguinalis* populations/that of the S population.

4.2. ACCase Activity

ACCase activities in the R and S populations changed similarly from 0 d to 10 d (Figure 1). The results showed that ACCase activities in both the R and S populations increased significantly at 1 d and peaked at 3 d. The activity in both the R (15HN10, 15HN1) and S populations declined at 3 d after treatment with haloxyfop-P-methyl and afterward tended to remain stable. However, ACCase activities in the two R populations were significantly higher than that of S populations during the whole experiment.

![Figure 1. Activities of acetyl-CoA carboxylase in resistant (15HN10, 15HN1) and susceptible (S) *Digitaria sanguinalis* populations.](image)

4.3. GST Activity

GST activity was analyzed over a period of 9 d (Figure 2). After haloxyfop-P-methyl treatment, the GST relative activity of the R (15HN10, 15HN1) and S populations increased and peaked first at 3 d and 2 d, then fell to the bottom at 4 d. A second peak was found at 7 d. The trend of GSTs in the three populations was basically the same. GST relative activity in the R populations was consistently greater than that in the S population.
4.4. NADPH Activity

NADPH relative activity was analyzed during a period of 10 d (Figure 3). After haloxyfop-P-methyl treatment, the NADPH relative activity of the 15HN10, 15HN1 and S populations increased and peaked at 3 d, 2 d and 3 d, respectively. Variation tendency in all the R and S populations changed similarly from 4 d to 10 d, while the activity in the S population was consistently greater than that in the R populations. The results indicated that there was no direct correlation between NADPH and metabolic resistance to haloxyfop-P-methyl in *D. sanguinalis*.
phytotoxicity may be present in the resistant tissues compared to the susceptible ones. Therefore, the R populations can survive more than S ones at the same applied herbicide doses, showing haloxyfop-P-methyl resistance in resistant *Digitaria sanguinalis* populations.

![Figure 4](image_url). Relative activities of carboxylesterase in resistant (15HN10, 15HN1) and susceptible (S) *Digitaria sanguinalis* populations.

5. Discussion

The survey of haloxyfop-P-methyl resistance in *D. sanguinalis* in the main cotton production regions in China revealed an increasing tendency. As a result, 57 *D. sanguinalis* populations have evolved different levels of resistance (2 ≤ RI ≤ 20) to haloxyfop-P-methyl. The continuous monitoring in Hunan and Hebei populations reflected a rapid spread of field-evolved resistance. *D. sanguinalis* has developed resistance primarily to haloxyfop-P-methyl. The major reason is that the excellent selectivity and high efficacy of haloxyfop-P-methyl made it an attractive choice for weed control in China’s cotton fields, leading to over-reliance on haloxyfop-P-methyl and, ultimately, strong selection pressures for *D. sanguinalis*. Although no high resistance (RI > 20) was found in this survey, more attention should be paid to delay the evolution of resistance.

Different levels of haloxyfop-P-methyl resistance in eight different cotton production provinces may be related to the integrated practices (such as chemical, cultural, biological and physical strategies) for managing weeds adopted by Chinese farmers. Regarding the evolution of weeds resistant to herbicides, TSR and NTSR are widely recognized as the two major explanations. ACCase is the target site for haloxyfop-P-methyl. ACCase activity is inhibited under haloxyfop-P-methyl stress. However, our results showed that ACCase activities both in the R (15HN10, 15HN1) and S populations rose continuously from 1 d to 3 d due to the application of haloxyfop-P-methyl (Figure 1). This might be caused by the plant’s stress response and rapid metabolism of the herbicide. Tang et al. also reported similar increase in ACCase activity from 1 d to 3 d in common roegneria (*Roegneria kamoji* Ohwi) [43]. Although variation tendency in the R and S populations was generally similar, the results also showed that the ACCase activity in the S population was consistently significantly lower than that in the R population. Lower ACCase activity leads to a reduced synthesis of branched-chain amino acids, which eventually causes plant death. The response of ACCase activity from the R and S accessions of *D. sanguinalis* was consistent at the whole-plant level. Compared to population 15HN10, population 15HN1 had higher RI but lower ACCase activity, which might be caused by TSR. Whether there was any gene amplification of target enzymes or ACCase mutation in the R seedlings requires further study.
The GST relative activity in all populations increased rapidly after treatment, which confirmed that plants express GSTs in response to numerous endogenous and xenobiotic stresses. In this paper, the GST relative activity in the S populations was found to be lower than that in R populations during the whole process (Figure 2). The increase in GSTs might be caused by a plant’s own stress response and rapid metabolism of the herbicide. In addition, the first increase was consistent with the report in quizalofop-P-ethyl-resistant *Eleusine indica*, while the second increase was also similar to that reported in haloxyfop-P-methyl-resistant *Eleusine indica* [40,44]. The significant differences in GST activity between the R and S populations indicated that enhanced metabolism mediated by GSTs is involved in haloxyfop-P-methyl resistance. The functions of P450s can be summarized as involving either hydroxylation or dealkylation. Whether the resistance of *D. sanguinalis* to haloxyfop-P-methyl was dominated by GST-mediated metabolism or other NTSR mechanisms requires further study.

NADPH-P450 reductase activates and inserts an atom from molecular oxygen to form a more reactive product using electrons from NADPH [10]. The NADPH relative activities were elevated in 15HN1, 15HN10 and S populations after herbicide application, but NADPH relative activity was significant higher in the susceptible *D. sanguinalis* than that in the two resistant weeds (Figure 3). Thus, in R populations, TSR could exist and result in different levels of NADPH activity. Whether NADPH was the candidate metabolic enzyme in *D. sanguinalis* conferring haloxyfop-P-methyl resistance or not requires further work. A previous study reported that CarE could activate AOPP graminicides in *Alopecurus myosuroides* by hydrolyzing the ester forms to their acid active forms [37]. Our results revealed that CarE from the S population was induced after haloxyfop-P-methyl application (Figure 4). We found that a much smaller change in CarE activity occurred in the R populations, which led to much less haloxyfop acid in the R tissues compared to the S ones. Results showed that CarE is highly related to NTSR in *D. sanguinalis*. Additional work needs to perform to elucidate the resistance mechanisms by biochemical and molecular approaches in the near future.

Efficiency associated with the chemical control of weeds has caused drawbacks due to herbicide resistance [45]. Nine blackgrass (*Alopecurus myosuroides*) populations from European fields were found to be resistant to fenoxaprop-P-ethyl [46]; *Eleusine indica* (L.) Gaertn. and *Setaria faberi* Herrm. were found to have evolved resistance to fluzifop-P-butyl and fenoxaprop-P-ethyl [12,47,48]. In China, shortawn foxtail (*Alopecurus aequalis* Sobol.) from wheat fields developed resistance to fenoxaprop-P-ethyl and haloxyfop-P-methyl [39,49]; *Roegneria kamoji* in wheat field evolved resistance to fenoxaprop-P-ethyl and clodinafop-propargyl [43]; *Eleusine indica* from cotton fields showed resistance to quizalofop-P-ethyl and haloxyfop-P-methyl [40,44]; Asia minor bluegrass (*Polypogon fugax*) from wheat fields was also reported to be resistant to fenoxaprop-P-ethyl [16]. Target-site genes and non-target-site genes have led to the evolution of resistance, and weeds can accumulate resistance genes [10]. NTSR of *Alopecurus japonicus* in China commenced with the report of developing resistance to PSII-inhibiting herbicides in 1990 [50]. The resistance mechanism of *Polypogon fugax* to fenoxaprop-P-ethyl exhibited both TSR and NTSR [16]. Han et al. confirmed that 70% of the tested rigid ryegrass (*Lolium rigidum*) populations, which were resistant to ACCase-inhibiting herbicides, contained both enhanced metabolism and ACCCase mutations [51]. Reports also proved that NTSR occurred in barnyard grass (*Echinochloa crusgalli* (L.) Beauv.), blackgrass (*Alopecurus myosuroides*), Italian ryegrass (*Lolium multiflorum*) and wild oat (*Avena* spp.) [52–56]. Thus, further studies are needed to determine whether TSR or NTSR plays a major role in resistance of *D. sanguinalis* to haloxyfop-P-methyl. Additional work will be performed to elucidate the resistance mechanisms by biochemical and molecular approaches in the near future.

6. Conclusions

According to the results from a nation-wide, long-term resistance monitoring effort, *D. sanguinalis* has developed resistance primarily to haloxyfop-P-methyl in China’s cotton
fields, exhibiting a rapid spread of field-evolved resistance. Enzyme activity assays showed the potential involvement of NADPH, CarE and GSTs in *D. sanguinalis* to haloxyfop-P-methyl resistance.

**Supplementary Materials:** The following are available online at [https://www.mdpi.com/article/10.3390/agronomy12051071/s1](https://www.mdpi.com/article/10.3390/agronomy12051071/s1), Table S1: Geographical origin of *D. sanguinalis* populations (2014); Table S2: Geographical origin of *D. sanguinalis* populations (2015); Table S3: Geographical origin of *D. sanguinalis* populations (2016); Table S4: Geographical origin of *D. sanguinalis* populations (2017).

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