Multiple resistance to ACCase and ALS-inhibiting herbicides in *Beckmannia syzigachne* (Steud.) Fernald without mutations in the target enzymes

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ABSTRACT

American slough grass (*Beckmannia syzigachne* [Steud.] Fernald) is a worldwide weed and is widely distributed in rice-wheat rotations in China. Fenoxaprop-*P*-ethyl and mesosulfuron-methyl are two major herbicides used to control *B. syzigachne*. Resistance has evolved in *B. syzigachne* under continuous selective pressure from herbicides. This study aimed to establish the cross-resistance pattern of a resistant population and explore the potential non-target-site based resistance mechanisms of *B. syzigachne*. Sequencing of target enzyme genes (acetyl coenzyme A carboxylase [ACCase] and acetolactate synthase [ALS]) revealed that there were no resistance-endowing amino acid substitutions in the resistant *B. syzigachne* population (R_{F1}) compared with the sensitive population (S_{F1}), and obtained the purified materials. Furthermore, piperonyl butoxide (PBO) and malathion showed synergistic effects with fenoxaprop-*P*-ethyl and mesosulfuron-methyl respectively in R_{F1}. Therefore, we speculate that the resistance observed in *B. syzigachne* was related with metabolic, mostly involving the cytochrome P450 enzymes. Cross resistance patterns showed that the purified resistant *B. syzigachne* produce high resistance to fenoxaprop-*P*-ethyl and pyroxsulam; intermediate resistance to flucarbazone-sodium; low resistance to quizalofop-*P*-ethyl, clodinafop-propargyl, sethoxydim and mesosulfuron-methyl; sensitive to clethodim pinoxaden and isoproturon.

Key words: Herbicide resistance, metabolic resistance, P450 inhibitors, target enzymes sequencing.

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INTRODUCTION

Agricultural weeds are mostly wild plant species, which thrive in agricultural ecosystems, competing for water, light and space with crops, and some are even intermediate hosts of diseases and pests. American slough grass (*Beckmannia syzigachne* [Steud.] Fernald) is adapted to the northern United States, southern Canada and part of eastern Asia (GBIF, 2017). In China, it is a troublesome weed which widely distributed in the wheat (*Triticum aestivum* L.) or oilseed rape (*Brassica rapa* L. subsp. *oleifera* (DC.) Metzg.) fields rotated with rice (*Oryza sativa* L.), mainly in the Yangtze Delta and the southwestern region (Li et al., 2013).

Herbicides are a major tool for controlling weeds to protect crop yields. Fenoxaprop-*P*-ethyl, belonging to the acetyl coenzyme A carboxylase (ACCase; EC 6.4.1.2) inhibitors, has long been used to selectively control grass weeds in wheat fields in China. However, the susceptibility of *B. syzigachne* to fenoxaprop-*P*-ethyl has decreased after many years use, and the resistance of *B. syzigachne* to fenoxaprop-*P*-ethyl was first reported in 2008 (Liu and Zhang, 2008). Consequently, mesosulfuron-methyl, a widely used acetolactate synthase (ALS; EC 4.1.3.18) inhibitor, rapidly became the most important herbicide for controlling ACCase inhibitor-resistant grass weeds (Bi et al., 2013). Mesosulfuron-methyl is well-known for its high efficiency, low animal toxicity worldwide and a broad herbicidal spectrum (Powles and Yu, 2010). However, resistance to ALS-inhibiting herbicides in *B. syzigachne* started to appear after several years of successful control (Li et al., 2015). Therefore, *B. syzigachne* became the most economically important crop weed, and the widespread evolution of multiple-herbicide resistance in this species makes its control more difficult.

There are generally two primary mechanisms in resistant weeds (Délye, 2005): target-site resistance (TSR) and non-target-site resistance (NTSR). TSR is due to changes in herbicide-binding sites or differences in the expression of target enzymes and has been well described (Délye, 2005; Powles and Yu, 2010). For example, the ACCase-inhibiting herbicides resistance is mainly caused by alterations of the ACCase enzyme. To date, 12 amino acid substitutions (AASs) at seven positions (i.e. 1781, 1999, 2027, 2041, 2078, 2088, and 2096) in the CT domain are implicated in conferring resistance to ACCase-inhibiting herbicides (Powles and Yu, 2010; Beckie and Tardif, 2012; Kaundun, 2014). Different ACCase mutations may confer distinct cross-resistance patterns to ACCase-inhibitors (Beckie and Tardif, 2012), and the homo/heterozygous status of the plants for

the mutation can also influence the resistance levels (Délye, 2005; Scarabel et al., 2011). The substitutions at Leu1781 can confer resistance to aryloxyphenoxypropionates (APPs), cyclohexanediones (CHDs) and pinoxaden (Délye et al., 2002), 2027Cys and 2041Asn alleles can cause resistance to APPs and pinoxaden, but not to CHDs (Délye et al., 2003; Petit et al., 2010; Scarabel et al., 2011); 2096Ala allele confers resistance mainly to APPs (Délye, 2005). For the target site ALS gene mutations, at least 28 resistance endowing AASs at eight positions of the ALS gene have been identified over the past two decades (i.e. 122, 197, 205, 376, 377, 574, 645 and 653) (Yu and Powles, 2014a; Heap, 2017). Generally, substitutions at Ala122 confer resistance to Imidazolinones (IMIs) herbicides, but not to Sulfonylureas (SUs) herbicides; substitutions at Pro197 confer resistance to SU herbicides, but not to IMI herbicides; and substitutions at Trp574 provide resistance to both SU and IMI herbicides (Yu and Powles, 2014a).

NTSR is a complicated phenomenon compared with the TSR. It is thought to be caused by constitutive or herbicide-induced mechanisms and therefore mostly be driven by inheritable differences in the expression of a set of genes (Dinelli et al., 2006; Délye, 2013). To date, there are four well-established gene families participating in NTSR: the cytochrome P450 monooxygenases (P450s), glutathione *S*-transferases (GSTs), glycosyltransferases (GTs) and ATP-binding cassette (ABC) transporters (Yuan et al., 2007). The P450s constitute the largest family of enzymatic proteins in higher plants and protect plants from harmful exogenous chemicals, including pesticides and industrial pollutants, making them less phytotoxic. P450s are reported to play a primary role in phase I herbicide metabolism in weeds and crops (Werck-Reichhart et al., 2000; Siminszky, 2006), and has often been detected using specific P450 inhibitors, such as the organophosphate insecticide malathion, 1-aminobenzotriazole and piperonyl butoxide (PBO) (Werck-Reichhart et al., 2000). There were reports revealed that P450 inhibitors could enhance the rate of metabolism of several herbicides and had been confirmed in some herbicide-resistant weeds (Preston, 2004; Yun et al., 2005; Yasuor et al., 2009).

However, there were no reports about metabolic resistance in fenoxaprop-*P*-ethyl and mesosulfuron-methyl resistant *B. syzigachne* without any AASs in the target enzymes genes. The objective of this research was (1) to clone and compare the carboxyl transferase (CT) domain of the ACCase gene and the ALS gene in fenoxaprop-*P*-ethyl-resistant or sensitive and mesosulfuron-methyl-resistant or sensitive *B. syzigachne*, (2) to obtain the offspring of resistant population (R_{F1}) and offspring of sensitive population (S_{F1}) which sharing homogenized genetic backgrounds, (3) to investigate the effects of P450 inhibitors on the dose response to fenoxaprop-*P*-ethyl and mesosulfuron-methyl in the specific resistant *B. syzigachne*, (4) to establish more accurate cross resistance patterns at plant level including six ACCase inhibitors, three ALS inhibitors and one photosystem II inhibitor in the R_{F1}.

MATERIALS AND METHODS

Plant material and herbicides

A suspected resistant population (R) was collected from wheat fields under a rice-wheat rotation field where fenoxaprop-*P*-ethyl and mesosulfuron-methyl had been used annually for several years in Anhui province, China. A suspected sensitive population (S) was collected from uncultivated land in Shandong province, China. To ensure the variability in field collection, each seed sample was collected from approximately 30 mature plants. Seed samples were air-dried and stored in sealed paper bags at 4 °C until used.

The herbicides used for dose response tests were: fenoxaprop-*P*-ethyl ((*R*)-2-[4-(6-chlorobenzoxazol-2-yloxy) phenoxy]propionic acid; 69 g L^{-1} EW, Bayer, Hangzhou, China); quizalofop-*P*-ethyl ((*R*)-2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy]propionic acid; 50 g L^{-1} EC, LIER-Chemical, Mianyang, China); clodinafop-propargyl (prop-2-ynyl (*R*)-2-[4-(5-chloro-3-fluoro-2-pyridyloxy) phenoxy]propionate; 15% WP, Jinr, Shandong, China); sethoxydim ((5*RS*)-2-[(*EZ*)-1-(ethoxyimino)butyl]-5-[(2*RS*)-2-(ethylthio)propyl]-3-hydroxycyclohex-2-en-1-one 12.5% EC, Cynda, Jinan, China); clethodim ((5*RS*)-2-{(1*EZ*)-1-[(2*E*)-3-chloroallyloxyimino]propyl}-5-[(2*RS*)-2-(ethylthio)propyl]-3-hydroxycyclohex-2-en-1-one; 120 g L^{-1} EC, Cynda, Jinan, China); pinoxaden (8-(2,6-diethyl-*p*-tolyl)-1,2,4,5-tetrahydro-7-oxo-7*H*-pyrazolo[1,2-*d*] [1,4,5]oxadiazepin-9-yl 2,2-dimethylpropionate; 5% EC, Syngenta, Suzhou, China); mesosulfuron-methyl (methyl 2-[(4,6-dimethoxypyrimidin-2-ylcarbamoyl) sulfamoyl]-α-(methanesulfonamido)-*p*-toluate; 30 g L^{-1} OF, Bayer, Hangzhou, China); flucarbazone-sodium (sodium [(4,5-dihydro-3-methoxy-4-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl)carbonyl]{[2-(trifluoromethoxy)phenyl] sulfonyl}azanide; 70%, WG, Arysta, Shanghai, China); pyroxsulam (*N*-(5,7-dimethoxy[1,2,4]triazolo[1,5-a] pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)pyridine-3-sulfonamide; 7.5% WG, Dow AgroSciences, Shanghai, China); isoproturon (3-*p*-cumenyl-1,1-dimethylurea; 50% WP, Bianjing, Suzhou, China), piperonyl butoxide (PBO) (2-(2-butoxyethoxy)ethyl 6-propylpiperonyl ether; 97%, Aladdin, Shanghai, China) and malathion (*S*-1,2-bis(ethoxycarbonyl)ethyl *O*,*O*-dimethyl phosphorodithioate; 95%, Ningbo Sunjoy Cropscience, Ningbo, China).

Acquisition of purified R_{F1} and S_{F1}

Preliminary screening test. Prior to planting, seeds were first stored at -20 °C for 7 d and then deposited in Petri dishes containing two layers of filter paper soaked with about 5 mL distilled water. All of the Petri dishes were placed in chamber at 22/15 °C (12 h day/12 h night temperatures) under 75% RH. After germination, the seeds were sown in plastic pots and transferred to a glasshouse (temperature maintained at approximately 15-25 °C, with 63%-75% RH and natural sunlight) (Li et al., 2013). Seedlings were thinned to six evenly sized plants per pot and watered as needed. At the three to four leaf stage, seedlings

of suspected R population were sprayed with fenoxaprop-*P*-ethyl at 0, 62.1 (1×), 186.3 (3×) and 558.9 (9×) g ai ha^{-1}; mesosulfuron-methyl at 0, 5.25 (1/3×), 15.75 (1×) and 47.25 (3×) g ai ha^{-1}. While the seedlings of suspected S population were sprayed with fenoxaprop-*P*-ethyl at 0, 20.7 (1/3×), 62.1 (1×) and 186.3 (3×) g ai ha^{-1}; mesosulfuron-methyl at 0, 1.75 (1/9×), 5.25 (1/3×) and 15.75 (1×) g ai ha^{-1}. Spraying was performed using a compressed air, moving nozzle cabinet sprayer equipped with one flat-fan nozzle (9503EVS, TeeJet, Wheaton, Illinois, USA) at a height of 50 cm above the foliage, calibrated to deliver 450 L ha^{-1} at a pressure of 0.28 MPa. The treated plants were returned to the greenhouse and randomly placed on raised beds. At 21 d after treatment (DAT), the result was checked visually.

Amplification, cloning and sequencing of the target enzyme genes. Seeds of both populations were treated as described above, with three seedlings per pot, and each population containing four pots. Forty milligrams of shoot tissue from individual plants in each pot was mixed and frozen in liquid nitrogen for DNA extraction. Total DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Doyle, 1987). As this harvest only included a small part of the plant and the remaining plant tissue was left and transplanted into larger plastic pots to produce seeds that would later be used to produce the R_{F1} and S_{F1} generation. When they were ripe, seeds from the two populations (R_{F1} and S_{F1}) were harvested and stored at room temperature for 2-mo to enable after-ripening.

To identify the molecular mechanism of resistance to fenoxaprop-*P*-ethyl in *B. syzigachne*, the primers and procedure in Li et al. (2013) were used to amplify the carboxyltransferase (CT) domain of the ACCase gene of *B. syzigachne* by polymerase chain reaction (PCR). In addition, the primers and procedure in Li et al. (2015) were used, which amplified a fragment including all known eight ALS inhibitor-resistant positions. The amplified DNA fragments were purified using the TIANgel Midi Purification Kit and cloned directly into pEASY-T1 vector (TransGen Biotech, Beijing, China). The sequences of the inserts were determined using the ABI PRISM 3730 DNA sequencer (Sangon Biotech, Shanghai, China). Twelve individual plants from the R population and six plants from the S population were sequenced and at least four positive clones from each biological replicate were sent for sequencing. Each fragment was sequenced in the forward and reverse directions by Shanghai Sangon Biological Engineering Technology & Services Co. (Shanghai, China). The sequencing data from the two *B. syzigachne* populations were compared to determine whether there were AASs associated with resistance using DNAMAN version 5.2.2 software (Lynnon Biosoft, Quebec, Canada).

To ensure the background of R_{F1} and S_{F1}, we randomly selected 60 plants from the R_{F1} and 30 plants from the S_{F1} sequenced as described above.

Sensitivity to fenoxaprop-*P*-ethyl and mesosulfuron-methyl following the P450s inhibitors

The seeds of R_{F1} and S_{F1} were pretreated as described above and then six seedlings were sown per pot. Seedlings at the three to four leaf stage were sprayed with PBO, fenoxaprop-*P*-ethyl, PBO plus fenoxaprop-*P*-ethyl, malathion, mesosulfuron-methyl and malathion plus mesosulfuron-methyl, in addition, we set up three concentration gradients of each P450 inhibitors (Table 1). There were a total 35 different treatments of each P450 inhibitors, including the blank control (spraying with the same volume of water). Spraying was performed as described above. At 21 d after treatment (DAT), the aboveground materials were harvested and the fresh-weight data were recorded.

PBO was applied in two applications of 2100 g ai ha^{-1} in 97 L ha^{-1} water, for total application of 4200 g ai ha^{-1} in 194 L ha^{-1} water. Malathion and PBO were formulated in a mixture of emulsifier (Tween-80, 1 mL L^{-1} water) and acetone and were applied 1 h prior to herbicide application (Preston et al., 1996). Reference plants were treated with a mixture of emulsifier and acetone. All treatments were replicated three times, and the experiment was conducted twice.

Sensitivity to other herbicides

Seedlings of R_{F1} and S_{F1} at the three to four leaf stage were sprayed with 10 herbicides at different rates (Table 2). Three weeks later, the results were checked as described above. All

Table 1. Piperonyl butoxide (PBO) and malathion treatment applied for P450 inhibitors.

Treatments	P450 inhibitors	Herbicides	
		R_{F1}	S_{F1}
	g ai ha^{-1}	g ai ha^{-1}	
PBO + Fenoxaprop-*P*-ethyl	0	0, 20.7, <u>62.1</u>, 186.3, 558.9, 1676.7, 5030.1	0, 0.77, 2.3, 6.9, 20.7, 62.1, 186.3
	Emulsifier + acetone	0, 20.7, 62.1, 186.3, 558.9, 1676.7, 5030.1	0, 0.77, 2.3, 6.9, 20.7, 62.1, 186.3
	1050	0, 20.7, 62.1, 186.3, 558.9, 1676.7, 5030.1	0, 0.77, 2.3, 6.9, 20.7, 62.1, 186.3
	2100	0, 20.7, 62.1, 186.3, 558.9, 1676.7, 5030.1	0, 0.77, 2.3, 6.9, 20.7, 62.1, 186.3
	<u>4200</u>	0, 20.7, 62.1, 186.3, 558.9, 1676.7, 5030.1	0, 0.77, 2.3, 6.9, 20.7, 62.1, 186.3
Malathion + Mesosulfuron-methyl	0	0, 1.75, 5.25, <u>15.75</u>, 47.25, 141.75, 425.25	0, 0.19, 0.58, 1.75, 5.25, 15.75, 47.25
	Emulsifier + acetone	0, 1.75, 5.25, 15.75, 47.25, 141.75, 425.25	0, 0.19, 0.58, 1.75, 5.25, 15.75, 47.25
	500	0, 1.75, 5.25, 15.75, 47.25, 141.75, 425.25	0, 0.19, 0.58, 1.75, 5.25, 15.75, 47.25
	<u>1000</u>	0, 1.75, 5.25, 15.75, 47.25, 141.75, 425.25	0, 0.19, 0.58, 1.75, 5.25, 15.75, 47.25
	2000	0, 1.75, 5.25, 15.75, 47.25, 141.75, 425.25	0, 0.19, 0.58, 1.75, 5.25, 15.75, 47.25

Underlined values are the recommended doses.
R_{F1}: Offspring of resistant *Beckmannia syzigachne* population; S_{F1}: offspring of sensitive *B. syzigachne* population.

Table 2. Herbicides treatments applied for dose-response tests.

Herbicides	R_{F1}	S_{F1}
	g ai ha^{-1}	
Fenoxaprop-*P*-ethyl	20.7, 62.1, 186.3, 558.9, 1676.7, 5030.1	0.77, 2.3, 6.9, 20.7, 62.1, 186.3
Quizalofop-*P*-ethyl	2.22, 6.67, 20.0, 60.0, 180.0, 540.0	0.74, 2.22, 6.67, 20.0, 60.0, 180.0
Clodinafop-propargyl	2.5, 7.5, 22.5, 67.5, 202.5, 607.5	0.83, 2.5, 7.5, 22.5, 67.5, 202.5
Sethoxydim	23.44, 46.88, 93.75, 187.5, 375.0, 750.0	11.72, 23.44, 46.88, 93.75, 187.5, 375.0
Clethodim	11.25, 22.5, 45.0, 90.0, 180.0, 360.0	5.62, 11.25, 22.5, 45.0, 90.0, 180.0
Pinoxaden	0.74, 2.22, 6.67, 20.0, 60.0, 180.0	0.25, 0.74, 2.22, 6.67, 20.0, 60.0
Mesosulfuron-methyl	1.75, 5.25, 15.75, 47.25, 141.75, 425.25	0.19, 0.58, 1.75, 5.25, 15.75, 47.25
Flucarbazone-sodium	10.5, 31.5, 94.5, 283.5, 850.5, 2551.5	1.17, 3.5, 10.5, 31.5, 94.5, 283.5
Pyroxsulam	3.0, 12.0, 48.0, 192.0, 768.0, 2304.0	0.25, 0.75, 3.0, 12.0, 36.0, 108.0
Isoproturon[a]	16.41, 32.81, 65.62, 131.25, 262.5, 525.0	8.20, 16.41, 32.81, 65.62, 131.25, 262.5

Underlined values are the recommended doses.
[a]Recommended dose for isoproturon was 1050 g ai ha^{-1}.
R_{F1}: Offspring of resistant *Beckmannia syzigachne* population; S_{F1}: offspring of sensitive *B. syzigachne* population.

treatments were replicated three times, and the experiment was conducted twice. Resistance to six ACCase-inhibiting herbicides, including three aryloxyphenoxypropionates (APPs), two cyclohexanediones (CHDs), and one phenylpyraxoline (DEN), at different doses was determined. Three ALS inhibitor and one photosystem II inhibitor were also tested in this experiment.

Statistical analyses

The results were expressed as percentage inhibition [(fresh weight of treated pots/fresh weight of control pots) × 100]. ANOVA was selected to determine whether results were significantly different between the data of the two runs of the experiment for each herbicide and was performed on all data using SPSS software (Version 20.0; IBM Corporation, Armonk, New York, USA). When the ANOVA showed nonsignificant difference between the two runs of the experiment, thus the data were pooled and used for subsequent analysis.

The herbicide rate that inhibited growth by 50% (GR_{50}) was estimated based on a four-parameter non-linear logistic model (sigmoidal logistic, four parameters) using SigmaPlot software (SigmaPlot v.12.0; Systat Software, San Jose, California, USA) (Hochberg et al., 2009):

$$Y = C + (D - C)/[1 + (x/GR_{50})^b]$$

In this model, C is the lower limit, D is the upper limit, GR_{50} is the effective dose causing 50% growth reduction, and b describes the slope of the GR_{50} curve. The fitted equation was used to estimate the GR_{50} value. The resistance index (RI) value was calculated by dividing the GR_{50} value of the resistant population by that of the sensitive population.

RESULTS

Preliminary screening test

The suspected R population could grow normally even at three times of the recommend dose of fenoxaprop-*P*-ethyl, while all the suspect S population dead at the recommend dose. For the mesosulfuron-methyl test, the suspect R individuals' growth was inhibited at the recommend dose, but all are survived; while for the suspect S population all dead at the recommend dose. The result of preliminary screening test showed that suspect R population did resistance to fenoxaprop-*P*-ethyl and mesosulfuron-methyl.

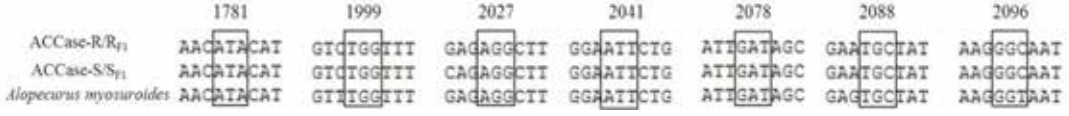
Sequencing and alignment of the target genes

A 1437 bp fragment including all seven ACCase inhibitor-resistant positions within the CT domain of ACCase gene was amplified, sequenced and blasted between the two populations and also between the R_{F1} and S_{F1}. A 1729 bp fragment encompassing all eight ALS inhibitor-resistant positions within the partial of the ALS gene was amplified to compare sequences between the two populations and also between the R_{F1} and S_{F1}. The blasted results indicated that there were not any reported AASs about resistance in the target enzymes genes in the R population as well as the R_{F1} (Figures 1 and 2).

Sensitivity to fenoxaprop-*P*-ethyl and mesosulfuron-methyl following the P450s inhibitors

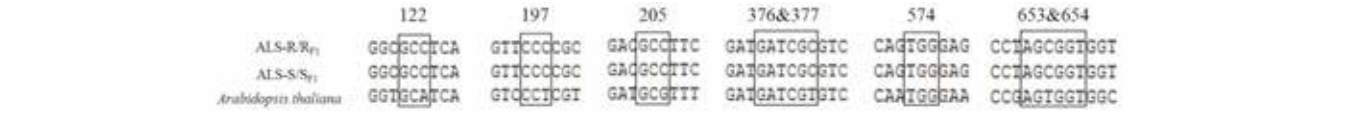
In the absence of fenoxaprop-*P*-ethyl, treatment with 4200 g ai ha^{-1} PBO had no obvious effect on the R_{F1} and S_{F1}, nor did malathion (2000 g ai ha^{-1}). Plants from the R_{F1} treated with fenoxaprop-*P*-ethyl plus PBO exhibited significantly increased mortality compared with fenoxaprop-*P*-ethyl treatment alone. Pretreatment with the maximum

Figure 1. Alignment of *Beckmannia syzigachne* ACCase with *Alopecurus myosuroides*. Boxes indicate amino acid positions whose substitutions are known to confer ACCase inhibitor resistance.



ACCase: Acetyl coenzyme A carboxylase; ACCase-R: CT domain of resistant population's ACCase gene; ACCase-S: CT domain of sensitive population's ACCase gene; R_{F1}: offspring of resistant *Beckmannia syzigachne* population; S_{F1}: offspring of sensitive *B. syzigachne* population.

Figure 2. Alignment of *Beckmannia syzigachne* ALS with *Arabidopsis thaliana*. Boxes indicate amino acid positions whose substitutions are known to confer ALS inhibitor resistance.



ALS: Acetolactate synthase; ALS-R: acetolactate synthase of resistant population; ALS-S: acetolactate synthase of sensitive population; R_{F1}: offspring of resistant *Beckmannia syzigachne* population; S_{F1}: offspring of sensitive *B. syzigachne* population.

concentration of PBO significantly inhibited growth of the R_{F1}, decreased the GR_{50} value by 60.2% compared with fenoxaprop-*P*-ethyl-only treatment. The malathion combined with mesosulfuron-methyl increased toxicity in plants, and pretreatment with malathion decreased the GR_{50} value by 65.6% compared with the mesosulfuron-methyl-only treatment in the R_{F1}. Moreover, we observed that mesosulfuron-methyl plus malathion or fenoxaprop-*P*-ethyl plus PBO also reduced the GR_{50} of the S_{F1} partly compared with herbicide treatment alone (Table 3 and Figure 3).

Levels of resistance to herbicides in R_{F1}

The results of whole-plant dose-response experiments showed that the R_{F1} produced high resistance to fenoxaprop-*P*-ethyl, based on the RI of GR_{50} which RI was as high as 31.2 (Table 4 and Figure 4) (Beckie and Tardif, 2012). The results showed that the R_{F1} exhibited low resistance to quizalofop-*P*-ethyl, clodinafop-propargyl and sethoxydim, with RI values of 2.8, 4.2 and 2.7, respectively. The R_{F1} displayed no resistance to clethodim and pinoxaden, with corresponding RIs below 2. Moreover, the R_{F1} showed multiple resistance to ALS inhibitors, but not including photosystem II inhibitors. The R_{F1} showed low resistance to mesosulfuron-methyl, intermediate resistance to flucarbazone-sodium, and high resistance to pyroxsulam, with RI values of 2.6, 7.7 and 23.6, respectively. The GR_{50} of R_{F1} to isoproturon was 63.3 g ai ha^{-1}, which value was much lower than the recommended rate (1050 g ai ha^{-1}) showing its high efficiency in controlling the multiple-resistant *B. syzigachne*.

Table 3. Response to herbicides following different concentrations of P450 inhibitors.

P450 inhibitors + herbicides	GR_{50}		RI
	R_{F1}	S_{F1}	
g ai ha^{-1}	g ai ha^{-1}		
Fenoxaprop-*P*-ethyl	324.8 ± 25.6	10.4 ± 1.6	31.2
1050 + fenoxaprop-*P*-ethyl	176.4 ± 11.6	7.0 ± 3.1	17.0
2100 + fenoxaprop-*P*-ethyl	153.1 ± 12.1	5.7 ± 0.6	14.7
4200 + fenoxaprop-*P*-ethyl	129.2 ± 14.3	5.2 ± 0.4	12.4
Mesosulfuron-methyl	27.6 ± 1.7	10.4 ± 3.8	2.6
500 + mesosulfuron-methyl	14.1 ± 0.6	8.4 ± 3.0	1.7
1000 + mesosulfuron-methyl	11.7 ± 1.1	7.5 ± 2.2	1.6
2000 + mesosulfuron-methyl	9.5 ± 0.3	5.6 ± 0.1	1.7

GR_{50}: Herbicide ratio required to decrease plant fresh weight by 50% compared to the untreated control. Each value represents the mean ± standard error; RI: Resistance index was calculated as the ratio between the GR_{50} of the resistant population and the GR_{50} of the susceptible population; R_{F1}: offspring of *Beckmannia syzigachne* resistant population; S_{F1}: offspring of sensitive *B. syzigachne* population.

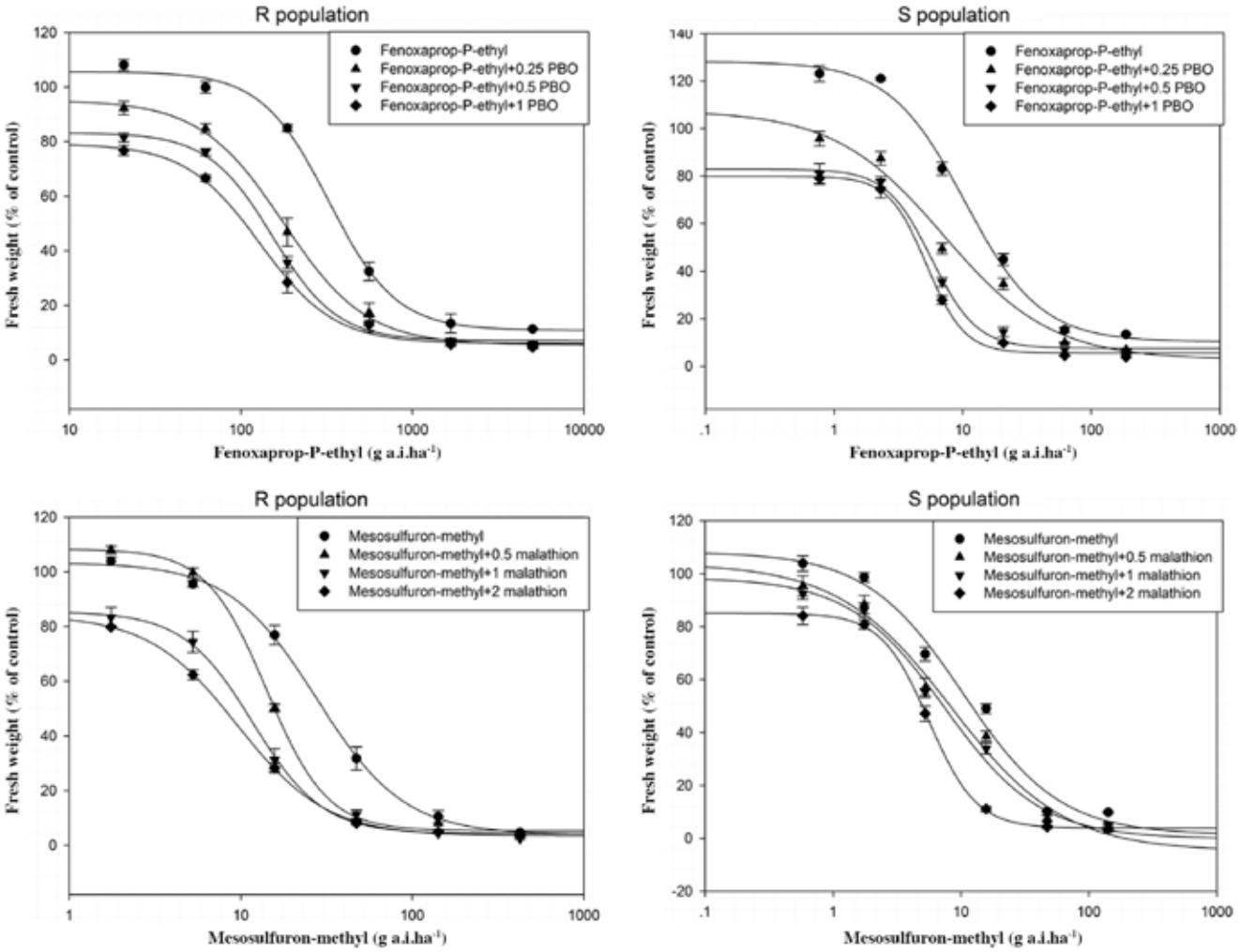
DISCUSSION

Metabolic resistance in weeds is much more complicated compared with TSR and due to the threat of NTSR to agriculture, extensive investigation of the underlying mechanism is necessary (Yu and Powles, 2014b). The population examined was shown to be resistant to fenoxaprop-*P*-ethyl and mesosulfuron-methyl though the preliminary screening test. The result of sequencing and alignment of the target genes showed that there were not any endow resistance AASs in the resistant population, neither in the R_{F1}. From above phenomenon, we suspect that NTSR is present in this specific resistant population. Therefore, we added an experiment using P450 inhibitors to verify this hypothesis and did a preliminary exploration about this kind of resistant *B. syzigachne*.

The result in our research also suggested that synergism may occur and there was an effect (particularly for the R_{F1}) when the PBO and malathion were added, which in accordance with a previous report (Pan et al., 2015). Moreover, we observed that mesosulfuron-methyl plus malathion or fenoxaprop-*P*-ethyl plus PBO also reduced the GR_{50} of the S_{F1} partly compared with herbicide treatment alone, probably at rates not sufficient to confer resistance though S_{F1} metabolizes some mesosulfuron-methyl or fenoxaprop-*P*-ethyl (Gaines et al., 2014). Based on above results, P450-based metabolism is most likely involved in the R_{F1}. There were several inhibitors of P450s had been identified, including 1-aminobenzo-triazole, tetcyclacis, PBO, carbon monoxide and tridiphane (McFadden et al., 1989; Zimmerlin and Durst, 1990; Siminszky, 2006; Pan et al., 2015). These inhibitors have provided evidence of the involvement of P450s in herbicide resistance and tolerance. Application of PBO to *Poa annua* in vivo showed that PBO exerted a synergistic effect with fenoxaprop-*P*-ethyl (Wang et al., 2013). Malathion has shown to present synergistic activity with the ALS-inhibiting herbicide chlorsulfuron against the resistant *Lolium rigidum* biotype (Preston et al., 1996). Similar effects of malathion on the metabolism of the ALS inhibitor propoxycarbazone-sodium have been reported for *Bromus tectorum* (Park et al., 2004).

P450-mediated mechanism can trigger resistance to herbicides with similar mechanisms or different mechanisms of action, including herbicides that have never been used. To obtain a more comprehensive understanding of the background of the R_{F1}, we characterized the cross and multiple resistance patterns to 10 herbicides at plant level. The evolution of multiple resistance to ACCase- and ALS-

Figure 3. Dose-response curve for above ground fresh weights of R_{F1} and S_{F1} plants treated with fenoxaprop-*P*-ethyl and mesosulfuron-methyl following the P450s inhibitors. Each point represents the mean of two experiments, each containing three replicates.



R_{F1}: Offspring of resistant *Beckmannia syzigachne* population; S_{F1}: offspring of sensitive *B. syzigachne* population; PBO: piperonyl butoxide.

inhibitors in the R_{F1} indicates a further threat to alternative herbicides for controlling the weed. Fortunately, there was no resistance to isoproturon, clethodim emulsifiable and pinoxaden, and then our results indicate that these four herbicides should be used. When managing this kind of resistant *B. syzigachne* with metabolic resistance, an integrated system should be used that includes rotation of crops and tillage systems as well as using a fully diversified herbicide program.

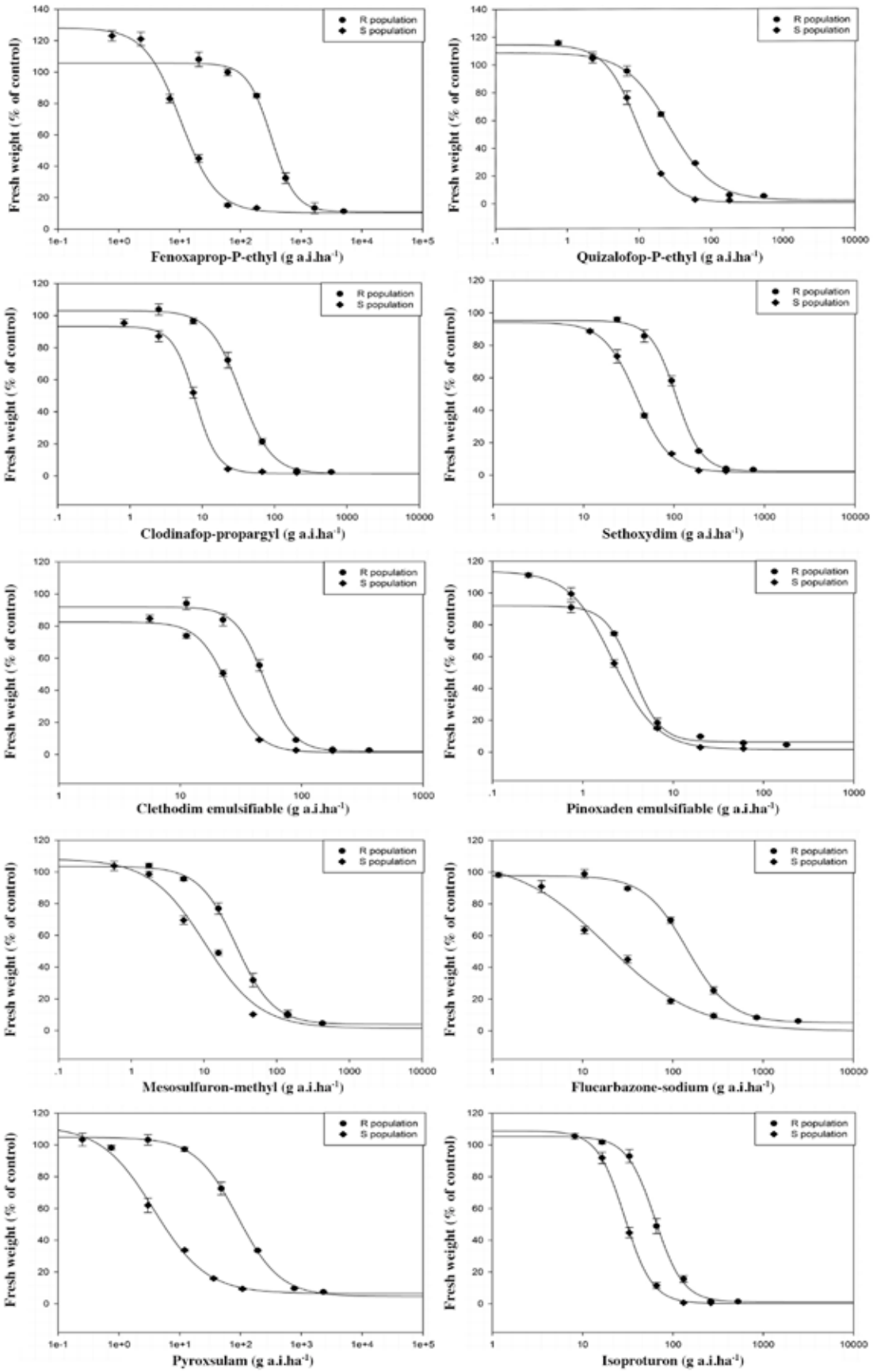
The results in this research were consistent with previous work showing that NTSR generally confers resistance to group of herbicides, or general for some herbicide groups (Yu and Powles, 2014b). The R_{F1} in this study was resistant to fenoxaprop-*P*-ethyl and mesosulfuron-methyl but without finding any AASs in the target enzymes. Therefore, we speculated that this might result from NTSR and/or other unknown mechanism and this inconclusive resistance mechanism may confer resistance in the R_{F1} and further analysis is in progress.

Table 4. The sensitivities of resistant and sensitive populations to other herbicides.

Herbicides	GR_{50}		RI
	R_{F1}	S_{F1}	
	g ai ha^{-1}		
Fenoxaprop-*P*-ethyl	324.8 ± 25.6	10.4 ± 1.6	31.2
Quizalofop-*P*-ethyl	26.0 ± 2.3	9.3 ± 0.6	2.8
Clodinafop-propargyl	33.7 ± 1.5	8.0 ± 0.4	4.2
Sethoxydim	105.4 ± 4.3	38.7 ± 1.5	2.7
Clethodim	49.3 ± 2.4	25.0 ± 1.6	1.9
Pinoxaden	3.6 ± 0.2	2.1 ± 0.03	1.7
Mesosulfuron-methyl	27.6 ± 1.7	10.4 ± 3.8	2.6
Flucarbazone-sodium	144.3 ± 10.7	18.7 ± 5.5	7.7
Pyroxsulam	89.5 ± 5.3	3.8 ± 0.8	23.6
Isoproturon	63.3 ± 3.2	29.2 ± 0.7	-

GR_{50}: Herbicide ratio required to decrease plant fresh weight by 50% compared to the untreated control. Each value represents the mean ± standard error; RI: Resistance index was calculated as the ratio between the GR_{50} of the resistant population and the GR_{50} of the susceptible population; R_{F1}: offspring of *Beckmannia syzigachne* resistant population; S_{F1}: offspring of sensitive *B. syzigachne* population.

Figure 4. Dose-response curve for above ground fresh weights of R_{F1} and S_{F1} plants treated with 10 herbicides. Each point represents the mean of two experiments, each containing three replicates.



R_{F1}: Offspring of resistant *Beckmannia syzigachne* population; S_{F1}: offspring of sensitive *B. syzigachne* population.

CONCLUSION

In conclusion, the two target enzymes were cloned and compared between the two *Beckmannia syzigachne* populations, resistant (R_{F1}) and sensitive (S_{F1}). The effects of P450 inhibitors on the dose response to fenoxaprop-*P*-ethyl and mesosulfuron-methyl in the R_{F1} were explored, as well as the comprehensive cross-resistance patterns. Further study is needed to determine whether resistance is associated with the expression level of the target enzymes. It is also possible that mutations in the upstream regulatory regions of the target genes influence resistance. Accordingly, these possibilities will be investigated in future studies.

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REFERENCES

Beckie, H.J., and Tardif, F.J. 2012. Herbicide cross resistance in weeds. Crop Protection 35:15-28.

Bi, Y.L., Liu, W.T., Li, L.X., Yuan, G.H., Jin, T., and Wang, J.X. 2013. Molecular basis of resistance to mesosulfuron-methyl in Japanese foxtail, *Alopecurus japonicus*. Journal of Pesticide Science 38:74-77.

Délye, C. 2005. Weed resistance to acetyl coenzyme A carboxylase inhibitors: an update. Weed Science 53:728-746.

Délye, C. 2013. Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade. Pest Management Science 69:176-187.

Délye, C., Wang, T., and Darmency, H. 2002. An isoleucine–leucine substitution in chloroplastic acetyl-CoA carboxylase from green foxtail (*Setaria viridis* L. Beauv.) is responsible for resistance to the cyclohexanedione herbicide sethoxydim. Planta 214:421-427.

Délye, C., Zhang, X.Q., Chalopin, C., Michel, S., and Powles, S.B. 2003. An isoleucine residue within the carboxyl–transferase domain of multidomain acetyl-coenzyme A carboxylase is a major determinant of sensitivity to aryloxyphenoxypropionate but not to cyclohexanedione inhibitors. Plant Physiology 132:1716-1723.

Dinelli, G., Marotti, I., Bonetti, A., Minelli, M., Catizone, P., and Barnes, J. 2006. Physiological and molecular insight on the mechanisms of resistance to glyphosate in *Conyza canadensis* (L.) Cronq. Pesticide Biochemistry and Physiology 86:30-42.

Doyle, J.J. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19:11-15.

Gaines, T.A., Lorentz, L., Figge, A., Herrmann, J., Maiwald, F., Mark-Christoph, O., et al. 2014. RNA-Seq transcriptome analysis to identify genes involved in metabolism-based diclofop resistance in *Lolium rigidum*. Plant Journal 78:865-876.

GBIF. 2017. Global Biodiversity Information Facility (GBIF), Madrid, Spain. Available at http://www.gbif.org (accessed February 2017).

Heap, I. 2017. The international survey of herbicide resistant weeds. Available at http://www.weedscience.org (accessed February 2017).

Hochberg, O., Sibony, M., and Rubin, B. 2009. The response of ACCase-resistant *Phalaris paradoxa* populations involves two different target site mutations. Weed Research 49:37-46.

Kaundun, S.S. 2014. Resistance to acetyl-CoA carboxylase inhibiting herbicides. Pest Management Science 70:1405-1417.

Li, L.X., Bi, Y.L., Liu, W.T., Yuan, G.H., and Wang, J.X. 2013. Molecular basis for resistance to fenoxaprop-P-ethyl in American sloughgrass (*Beckmannia syzigachne* Steud.). Pesticide Biochemistry and Physiology 105:118-121.

Li, L.X., Liu, W.T., Chi, Y.C., Guo, W.L., Luo, X.Y., and Wang, J.X. 2015. Molecular mechanism of mesosulfuron-methyl resistance in multiply-resistant American sloughgrass (*Beckmannia syzigachne*). Weed Science 63:781-787.

Liu, B.X., and Zhang, S.R. 2008. Studies on resistance of weeds *Beckmannia syzigachne* to the herbicide fenoxapro-*P*-ethyl. Jiangsu Journal of Agricultural Science 24:124-126.

McFadden, J.J., Frear, D.S., and Mansager, E.R. 1989. Aryl hydroxylation of diclofop by a cytochrome P450 dependent monooxygenase from wheat. Pesticide Biochemistry and Physiology 34:92-100.

Pan, L., Li, J., Zhang, T., Zhang, D., and Dong, L.Y. 2015. Cross-resistance patterns to acetyl coenzyme A carboxylase (ACCase) inhibitors associated with different ACCase mutations in *Beckmannia syzigachne*. Weed Research 55:609-620.

Park, K.W., Fandrich, L., and Mallory-Smith, C.A. 2004. Absorption, translocation, and metabolism of propoxycarbazone-sodium in ALS-inhibitor resistant *Bromus tectorum* biotypes. Pesticide Biochemistry and Physiology 79:18-24.

Petit, C., Bay, G., Pernin, F., and Délye, C. 2010. Prevalence of cross- or multiple resistance to the acetyl-coenzyme A carboxylase inhibitors fenoxaprop, clodinafop and pinoxaden in black-grass (*Alopecurus myosuroides* Huds.) in France. Pest Management Science 66:168-177.

Powles, S.B., and Yu, Q. 2010. Evolution in action: plants resistance to herbicides. Annual Review of Plant Biology 61:317-347.

Preston, C., 2004. Herbicide resistance in weeds endowed by enhanced detoxification: complication for management. Weed Science 52:448-453.

Preston, C., Tardif, F.J., Christopher, J.T., and Powles, S.B. 1996. Multiple resistance to dissimilar herbicide chemistries in a biotype of *Lolium rigidum* due to enhanced activity of several herbicide degrading enzymes. Pesticide Biochemistry and Physiology 54:123-134.

Scarabel, L., Panozzo, S., Varotto, S., and Sattin, M. 2011. Allelic variation of the ACCase gene and response to ACCase-inhibiting herbicides in pinoxaden-resistant *Lolium* spp. Pest Management Science 67:932-941.

Siminszky, B. 2006. Plant cytochrome P450-mediated herbicide metabolism. Phytochemistry Reviews 5:445-458.

Wang, H.C., Li, J., Li, B., Lou, Y.L., and Dong, L.Y. 2013. The role of cytochrome P450 monooxygenase in the different responses to fenoxaprop-P-ethyl in annual bluegrass (*Poa annua* L.) and short awned foxtail (*Alopecurus aequalis* Sobol.) Pesticide Biochemistry and Physiology 107:334-342.

Werck-Reichhart, D., Hehn, A., and Didierjean, L. 2000. Cytochromes P450 for engineering herbicide tolerance. Trends in Plant Science 5:116-123.

Yasuor, H., Osuna, M.D., Ortiz, A., Saldain, N.E., Eckert, J.W., and Fischer, A.J. 2009. Mechanism of resistance to penoxsulam in late watergrass [*Echinochloa phyllopogon* (Stapf) Koss.] Journal of Agricultural and Food Chemistry 57:3653-3660.

Yu, Q., and Powles, S.B. 2014a. Resistance to AHAS inhibitor herbicides: current understanding. Pest Management Science 70:1340-1350.

Yu, Q., and Powles, S.B. 2014b. Metabolism-based herbicide resistance and cross-resistance in crop weeds: a threat to herbicide sustainability and global crop production. Plant Physiology 166:1106-1118.

Yuan, J.S., Tranel, P.J., and Stewart, C.N. 2007. Non-target-site herbicide resistance: a family business. Trends in Plant Science 12:6-13.

Yun, M.S., Yogo, Y., Miura, R., Yamasue, Y., and Fischer, A.J. 2005. Cytochrome P-450 monooxygenase activity in herbicide-resistant and -susceptible late watergrass (*Echinochloa phyllopogon*). Pesticide Biochemistry and Physiology 83:107-114.

Zimmerlin, A., and Durst, F. 1990. Xenobiotic metabolism in plants Aryl hydroxylation of diclofop by a cytochrome P-450 enzyme from wheat. Phytochemistry 29:1729-1732.