Reversing resistance to tembotrione in an *Amaranthus tuberculatus* (var. *rudis*) population from Nebraska, USA with cytochrome P450 inhibitors

Maxwel C Oliveira,[a]* **Todd A Gaines,**[b] **Franck E Dayan,**[b] **Eric L Patterson,**[b] **Amit J Jhala**[c] **and Stevan Z Knezevic**[a]

Abstract

BACKGROUND: A population of *Amaranthus tuberculatus* (var. *rudis*) was confirmed resistant to 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor herbicides (mesotrione, tembotrione, and topramezone) in a seed corn/soybean rotation in Nebraska. Further investigation confirmed a non-target-site resistance mechanism in this population. The main objective of this study was to explore the role of cytochrome P450 inhibitors in restoring the efficacy of HPPD-inhibitor herbicides on the HPPD-inhibitor resistant *A. tuberculatus* population from Nebraska, USA (HPPD-R).

RESULTS: Enhanced metabolism via cytochrome P450 enzymes is the mechanism of resistance in HPPD-R. Amitrole partially restored the activity of mesotrione, whereas malathion, amitrole, and piperonyl butoxide restored the activity of tembotrione and topramezone in HPPD-R. Although corn was injured through malathion followed by mesotrione application a week after treatment, the injury was transient, and the crop recovered.

CONCLUSION: The use of cytochrome P450 inhibitors with tembotrione may provide a new way of controlling HPPD-inhibitor resistant *A. tuberculatus*, but further research is needed to identify the cytochrome P450 candidate gene(s) conferring metabolism-based resistance. The results presented here aid to gain an insight into non-target-site resistance weed management strategies.
© 2017 Society of Chemical Industry

Supporting information may be found in the online version of this article.

Keywords: amitrole; malathion; mesotrione; piperonyl butoxide; synergists; waterhemp; weed resistance management

1 INTRODUCTION

Waterhemp [*Amaranthus tuberculatus* (var. *rudis*)] is a troublesome broadleaf weed primarily found in corn (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.] production fields in the north–central USA. In the last 20 years, changes in cultural and weed management practices, including reduced reliance on soil-applied herbicides and the adoption of conservation farming tillage practices have resulted in weed shifts.[1,2] *Amaranthus tuberculatus* has shown a particular propensity to adapt to corn/soybean cropping systems.[1 3 4] Its small seed size, rapid growth rate, competitive ability, and capacity to tolerate water stress are major factors contributing to the rise of *A. tuberculatus* as a successful weed in the north–central USA.[5–8] Additionally, *A. tuberculatus* has a prolific seed production ability, as a single female plant can produce one million seeds.[9] As a dioecious species, *A. tuberculatus* is an obligate outcrosser, which favors genetic variability and increases its ability to evolve resistance by sharing resistance genes through pollination.[10–12] Therefore, the weed predominance in cropping systems with high selection pressure imposed by herbicides has resulted in the widespread occurrence of herbicide resistance in

A. tuberculatus. In the USA, there are 50 unique cases of single-, cross-, and multiple-resistance in *A. tuberculatus*.[13]

Herbicide resistance in *Amaranthus* species has evolved via both target-site resistance (TSR) and non-target-site resistance (NTSR) to six different herbicide site-of-action groups (SOA). TSR is well understood and is usually determined by dominant alleles at a single nuclear gene locus.[14,15] In *A. tuberculatus*, TSR can occur as a result of amino acid substitutions in the target

* Correspondence to: MC Oliveira, Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Concord, NE 68583, USA. E-mail: moliveira@huskers.unl.edu

Correction added on 5 October 2017, after first online publication: on Table 3, Aminotriozole was replaced by Amitrole.

a Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Concord, NE, USA

b Department of Bioagricultural Sciences and Pest Management, Colorado State University in Fort Collins, CO, USA

c Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, NE, USA

enzyme, codon deletion, gene amplification and/or overexpression of target protein.[16–18] By contrast, NTSR can be a result of herbicide differential translocation, sequestration or enhanced metabolism.[15,19] The NTSR to herbicides is typically a quantitative trait and is considered a major challenge for weed science in the next decades.[20]

The evolution of resistance to 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor herbicides is the latest case of resistance in *A. tuberculatus*.[21,22] This type of herbicide resistance has been reported in only two *Amaranthus* species. Two *A. palmeri* biotypes from Kansas and Nebraska evolved resistance to mesotrione via a metabolism-based mechanism, as well as increased gene transcription and protein expression.[23] The *A. palmeri* biotype from Nebraska was also resistant to tembotrione via enhanced metabolism.[24] The cytochrome P450 monooxygenase (P450) inhibitor malathion increased sensitivity to mesotrione in a resistant *A. tuberculatus* biotype from Illinois shown to have enhanced mesotrione metabolism.[25] Therefore, resistance mechanisms reported in *Amaranthus* species to HPPD-inhibitor herbicides are both metabolism based and/or a result of HPPD gene overexpression.

Understanding the mechanisms of herbicide resistance is important for recommending the best weed management strategies. The most common management recommendation for combating resistant weeds is the use of herbicide tank-mixtures, sequential herbicide applications (PRE followed by POST), and rotation of herbicides and crop traits with different SOA.[26–28] However, NTSR mechanisms can confer unpredictable cross-resistance to different herbicide SOA.[29] In the case of NTSR, the addition of P450- and glutathione *S*-transferase-inhibitors has also been reported as a potential strategy to delay metabolism-based resistance.[30–32]

A population of *A. tuberculatus* (HPPD-R) with resistance to POST application of three HPPD-inhibitor herbicides (mesotrione, tembotrione, and topramezone) was reported in a seed corn/soybean rotation field in northeast Nebraska.[33] This population was highly resistant to mesotrione (18×), followed by (fb) tembotrione (6×), and topramezone (2×). The mechanism of resistance to mesotrione in this HPPD-R population is enhanced herbicide metabolism.[34] Therefore, experiments were conducted in the field, greenhouse, and laboratory to determine if the mechanism of resistance was metabolism based via cytochrome 450 enzymes and to investigate the role of P450 inhibitor synergists in reversing resistance in HPPD-R. The hypothesis was that the combination of cytochrome P450 inhibitors with mesotrione, tembotrione, and topramezone would reverse HPPD-inhibitor resistance in HPPD-R to a susceptible phenotype.

2 MATERIAL AND METHODS

2.1 Plant material and growth conditions

2.1.1 Field studies

A field experiment was conducted in 2014 and 2016 at a Platte County field location near Columbus, NE, USA, where the HPPD-R was reported.[33] The soil type at the study location was a silty clay loam (12% sand, 60% silt, 28% clay) with 3.3% organic matter and a pH of 6.8. Glyphosate- and glufosinate-tolerant hybrid corn 'Golden Harvest H-9138' was seeded at 79 280 seeds ha⁻¹ in rows spaced 76 cm apart on 22 May 2014, and 20 May 2016. The experiment was arranged in a randomized complete block design with three replications with a plot size of 3 × 7.6 m. An individual plot was considered as an experimental unit. Monthly mean air

temperature and total precipitation data during the study periods are provided in Table S1.

2.1.2 Greenhouse studies

Two *A. tuberculatus* phenotype, the HPPD-R and an HPPD-susceptible (HPPD-S) were studied. The HPPD-R seeds were harvested from a field in Platte County, NE, USA with confirmed resistance in 2014, whereas HPPD-S *A. tuberculatus* seeds were harvested from a field in Dixon County, NE in 2014 with a known history of controlling *A. tuberculatus* with HPPD inhibitors. Seeds were cleaned and stored at 5 °C until used in the greenhouse study in 2015 and 2016 at the University of Nebraska-Lincoln. Seeds were planted in 900 cm³ plastic trays containing peat/soil/sand/vermiculite (4: 2: 2: 2) potting mix. Emerged seedlings (1 cm) were transplanted into 164 cm³ cone-tainers in 2015 or 713 cm³ plastic pots in 2016 containing the identical potting mix described above. Plants were supplied with adequate water and kept under greenhouse conditions at 28/20 °C day/night temperature with 80% relative humidity (RH). Plants were supplied nutrients twice a week with 3 mg of N/P₂O₅/K₂O (20-10-20 Peters® Professional, JR Peters Inc., Allentown, PA, USA) for each 100 cm³ of the potting mix until plants were 8–10 cm tall. Artificial lighting was provided using metal halide lamps (600 µmol photons m⁻² s⁻¹) to ensure a 15 h photoperiod.

2.2 Efficacy of cytochrome P450 inhibitor with HPPD-inhibitor for control of HPPD-R and HPPD-S

2.2.1 Dose–response of mesotrione with or without malathion

A dose–response study was conducted in 2014 under field conditions to evaluate the synergistic effect of malathion with mesotrione. Treatments were arranged in a factorial design with five mesotrione rates [0, 1× (105 g a.i. ha⁻¹), 2×, 4×, and 8×]; and two malathion rates (0 and 2000 g a.i. ha⁻¹). Malathion treatments were applied 2 h prior to mesotrione application.

Treatments were applied at the V3 corn stage (20–25 cm tall) and when the HPPD-R was 8–10 cm tall with a CO₂-pressurized backpack sprayer calibrated to deliver 140 L ha⁻¹ aqueous solution at 248 kPa with a 2 m spray boom through TeeJet® AIXR 110020 sprayer nozzles at a speed of 4.3 km h⁻¹. Corn injury was assessed at 7, 14, and 21 days after treatment (DAT) using a scale of 0 to 100% (where 0 is no injury and 100 is plant death), based on chlorosis, bleaching, and stunting compared with non-treated plants. The HPPD-R control was evaluated based on symptoms such as bleaching, necrosis, and stunting of plants compared with non-treated plants.

In the dose–response study, doses needed to reach HPPD-R 50% (ED₅₀) control and 10% (ED₁₀), 30% (ED₃₀), and 50% (ED₅₀) corn injury were determined using the symmetric three-parameter logistic model function (*l3*) of the drc package in R statistical software.[35]

$$Y = d \exp(-\exp(b(\log(x) - e)))　　　　(1)$$

In this model, *Y* is the HPPD-R control (%) or corn injury (%), *d* is the upper limit, and *e* (ED₅₀) represents the inflection point. The parameter *b* is the relative slope around parameter *e*, and *x* is mesotrione dose in g a.i. ha⁻¹.

The mesotrione (with and without malathion) ED₅₀ indices on HPPD-R control were compared using the EDcomp function of the drc package in R.[36] The EDcomp function compares the ED₅₀ ratio using *t*-statistics, where $P < 0.05$ indicates that ED₅₀ values are significantly different between treatments.

2.2.2 P450 inhibitor and herbicide efficacy under greenhouse conditions

The research was conducted in a greenhouse in 2015 at the University of Nebraska-Lincoln to evaluate the efficacy of a P450 inhibitor with HPPD-inhibitor for control of HPPD-R and HPPD-S.

Separate experiments were conducted for the HPPD-R and HPPD-S populations and repeated twice. Each experiment was arranged in a complete randomized design, and the experimental unit was a cone-tainer (164 cm³) with a single plant. Treatments were arranged in a factorial design with three P450 inhibitors [malathion, aminotriazole (hereafter referred amitrole), and piperonyl butoxide (PBO)] and three HPPD-inhibitors (mesotrione, tembotrione, and topramezone) with five replications. Preliminary dose–response studies were conducted to determine the PBO and amitrole rates on *A. tuberculatus* (Fig. S1). The malathion rate was based on another study with *A. tuberculatus*.[25]

The P450 inhibitor treatments included malathion (Malathion®, PBI-Gordon Corp., Kansas City, MO, USA) applied at 2000 g a.i. ha⁻¹; PBO (Syner Pro®, Syngenta Crop Protection, Research Triangle Park, NC, USA) applied at 2000 g a.i. ha⁻¹; amitrole (Amitrol 240®, NuFarm, Calgary, AB, Canada) applied at 13.1 g a.i. ha⁻¹, and a non-treated control. Cytochrome P450 inhibitors were sprayed 2 h prior to herbicide application. The HPPD-inhibitor herbicide treatments included mesotrione (Callisto®, Syngenta Crop Protection) applied at 105 g a.i. ha⁻¹ plus crop oil concentrate 1% v/v (Agri-Dex®, Helena Chemical Co., Collierville, TN, USA) plus ammonium sulfate at 20.5 g L⁻¹ (DSM Chemicals North America Inc., Augusta, GA, USA); tembotrione (Laudis®, Bayer Crop Science, Research Triangle Park, NC, USA) applied at 92 g a.i. ha⁻¹ plus methylated seed oil 1% v/v (Noble®, Winfield Solutions, Shoreview, MN, USA) plus 20.5 g L⁻¹ ammonium sulfate; and topramezone (Impact®, AMVAC, Los Angeles, CA, USA) applied at 24.5 g a.i. ha⁻¹ plus methylated seed oil 1% v/v and 20.5 g L⁻¹ ammonium sulfate; and a non-treated control. Two days after treatment, soil drenches of 5 mM malathion or PBO solutions were applied with a syringe in their respective treatments. The soil drench was performed only in this greenhouse study.

Herbicide treatments were applied to 8–10 cm tall HPPD-R and HPPD-S seedlings with a single-tip chamber sprayer (DeVries Manufacturing Corp., Hollandale, MN, USA). The sprayer had an 8001 E nozzle (Spraying Systems Co., Wheaton, IL, USA) calibrated to deliver 140 L ha⁻¹ spray volume at 210 kPa at a speed of 3.7 km h⁻¹. HPPD-R and HPPD-S control was assessed visually at 21 DAT using a scale of 0 to 100% (where 0 is no injury and 100 is plant death). Control ratings were based on symptoms such as bleaching, necrosis, and stunting of plants compared with non-treated plants. Aboveground biomass was harvested at 21 DAT from each experimental unit and oven-dried at 65 °C until reaching constant dry weight; then the biomass was recorded. The biomass (g) data were converted into biomass reduction (%) compared with the non-treated experimental unit as:

$$\text{HPPD-R or HPPD-S biomass reduction } (\%) = \left[\left(\bar{E} - B \right) / \bar{E} \right] * 100 \tag{2}$$

where $\bar{E}$ is the mean biomass (g) of the non-treated experimental unit, and B is the biomass (g) of an individual treated experimental unit.

Analysis of variance (ANOVA) was performed using PROC GLIMMIX in SAS version 9.4 (SAS Institute Inc. Cary, NC, USA). Control (%) and biomass reduction (%) data were analyzed with beta distribution with ilink function to meet assumptions of residual variance analysis. If ANOVA indicated significant treatment effects, means were separated at $P < 0.05$ with Fisher's protected LSD test. The results were presented separately for each herbicide.

2.2.3 P450 inhibitor and herbicide efficacy under field conditions

In 2016, a field study was conducted with the same set of treatments as described in the greenhouse efficacy study. The objective was to evaluate the effects of P450s and HPPD-inhibitors herbicide under field conditions.

Treatments were applied at V4 corn stage (25–30 cm tall) and when the HPPD-R was 8–10 cm tall. Herbicide application, assessment of corn injury and HPPD-R control was similar to that described in the dose–response study (Section 2.2.1).

For the study in 2016, the ANOVA was performed using PROC GLIMMIX in SAS, similar to that previously demonstrated in the greenhouse study (Section 2.2.2), but block was treated as a random effect. The statistical analysis on corn injury was not performed due to the insignificant crop injury.

2.3 LC/MS–MS analysis of mesotrione and tembotrione metabolism in HPPD-R and HPPD-S leaves

### 2.3.1 Herbicide application and plant harvest

The research was conducted in a greenhouse in 2016 at the University of Nebraska-Lincoln as the first part of LC/MS–MS analysis. The treatments and experimental design were the same as described in the greenhouse efficacy study, but with 20 replications. The experimental unit was 713 cm³ plastic pots with three *A. tuberculatus* plants (HPPD-R or HPPD-S). Herbicide treatments were applied similarly to the greenhouse efficacy study. At 12, 24, 72, 168, and 336 hours after treatment (HAT), four random replications of each treatment were harvested at 1 cm above the plant cotyledons. Leaf material was stored in Falcon tubes (Falcon™ 50 ml Conical Centrifuge Tubes, ThermoFisher Scientific, Waltham, MA, USA) at −80 °C until used in the LC–MS/MS system Nexera X2 (Shimadzu Scientific Instruments, Columbia, MD, USA). For the LC–MS/MS analysis, only treatment combinations of P450 inhibitors (malathion, amitrole, PBO, and non-treated control) with mesotrione and tembotrione applied on the HPPD-R, and mesotrione and tembotrione applied on the HPPD-S were used. Topramezone was not studied in the LC–MS/MS due to the relatively lower resistance level (2×).

2.3.2 HPPD-R and HPPD-S leaf extraction

The HPPD-R and HPPD-S leaf fresh weights were determined by weighing the Falcon tubes before and after herbicide extraction from leaves. The treated leaves of each replication were washed and centrifuged (Sorvall™ Legend™ XT/XF, ThermoFisher Scientific) at 5000 *g* for 15 min in 20 ml washing buffer containing 20% (v/v) methanol. The supernatants were discarded, and leaf tissue was extracted with 20 ml of 90% (v/v) ethanol. The ethanol and leaf tissue was homogenized (PowerGen 125 Laboratory Homogenizer, Fisher Scientific, Waltham, MA, USA) for 30 s. Then the solution was centrifuged for 15 min at 10 000 *g* (Sorvall™ Legend™ XT/XF, ThermoFisher Scientific). The supernatants were transferred to 5 ml vials (Shimadzu™ Autosampler Vials, Shimadzu Scientific Instruments) and vials were stored at 5 C until used in the LC–MS/MS analysis.

2.3.3 Identification of mesotrione and tembotrione in HPPD-R and HPPD-S leaves

LC–MS/MS system consisted of a Nexera X2 UPLC with 2 LC-30 AD pumps, an SIL-30 AC MP autosampler, a DGU-20A5 Prominence
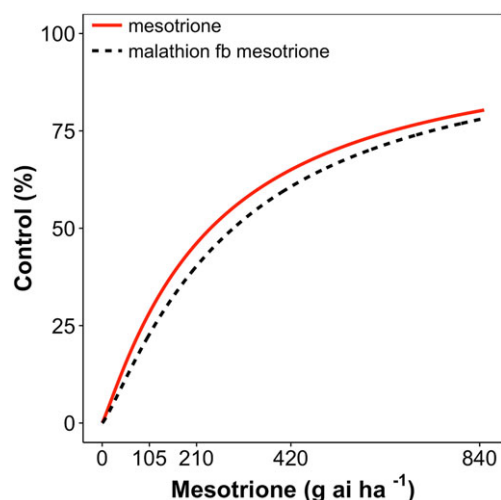
Figure 1. Dose–response of mesotrione with or without 2000 g a.i. ha^{-1} of malathion on 8–10 cm tall HPPD-inhibitor herbicide resistant *Amaranthus tuberculatus* (HPPD-R) control (%) 21 days after treatment in a field study in 2014 near Columbus, Platte County, NE, USA. fb, followed by.

Table 1. Estimated parameters from the dose–response of mesotrione with or without 2000 g a.i. ha^{-1} of malathion on 8–10 cm tall HPPD-inhibitor herbicide resistant *Amaranthus tuberculatus* (HPPD-R) control (%) 21 days after treatment in a field study in 2014 near Columbus, Platte County, NE, USA

	HPPD-R control (%)			
	Parameters[b]			
Treatment[a]	b (±SE)	d (±SE)	e (ED$_{50}$) ± SE	P-value[c]
	(%)		(g a.i. ha^{-1})	
mesotrione	−1.1 (0.1)	100	241 (28)	0.16
malathion fb mesotrione	−1.1 (0.1)	100	292 (23)	

[a] Mesotrione alone and malathion followed by (fb) mesotrione on the HPPD-inhibitor herbicide resistant *Amaranthus tuberculatus* (HPPD-R) population in a field at Platte County, NE in 2014.
[b] b, the slope; d, the upper limit (locked at 100); and e (ED$_{50}$), the inflection point relative to the upper limit. The ED$_{50}$ is an effective dose of mesotrione needed to reach 50% HPPD-R control. SE, standard error.
[c] Mesotrione vs. malathion followed by mesotrione on HPPD-R t-statistics comparison of e (ED$_{50}$), $P > 0.05$ indicates a non-significant difference between treatments.

degasser, a CTO-30A column oven, and SPD-M30A diode array detector coupled to an 8040-quadrupole mass-spectrometer.

For mesotrione (technical grade), the MS was in positive mode with an MRM optimized for 340.1 > 227.95 and set for a 100 ms dwell time with a Q1 pre-bias of −16.0 V, a collision energy of −18.0 V and a Q3 pre-bias of −16.0 V. Samples were chromatographed on a 100 × 4.6 mm Phenomenex kinetex 2.6 µm biphenyl column maintained at 40 °C. Solvent A consisted of water with 0.1% formic acid, and solvent B was methanol with 0.1% formic acid. The solvent program started at 50% B and increased to 70% B by 8 min and 90% B by 11 min. It was maintained at 90% B for 2 min. The solvent was returned to 50% B and maintained there for 3 min before the next injection. The flow rate was set at 0.4 ml min^{-1}, and each sample was analyzed as 1 µl injection volumes.

For tembotrione (technical grade), the MS was in negative mode with an MRM optimized for 439.1 > 226.05 and set for a 100 ms dwell time with a Q1 pre-bias of 11.0 V, a collision energy of 11.0 V and a Q3 pre-bias of 14.0 V. The samples were chromatographed on a 100 × 4.6 mm Phenomenex kinetex 2.6 µm biphenyl column maintained at 40 °C. Solvent A consisted of water with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid. The solvent program started at 80% B and was increased to 100% B in 3.5 min and then maintained at 100% for 2 min. The solvent was returned to 80% B and maintained there for 3 min before the next injection. The flow rate was set at 0.4 ml min^{-1}, and each sample was analyzed as 1 µl injection volumes.

The total amount of herbicide was expressed in leaf fresh weight (µg herbicide g^{-1} fresh weight). The amount of herbicide (µg herbicide g^{-1} fresh weight) was converted into herbicide metabolism (%) compared with the content of herbicide at 12 HAT (maximum herbicide absorption) as:

$$\text{Herbicide Metabolism } (\%) = \left[\left(\overline{Y} - C \right) / \overline{Y} \right] \times 100 \quad (3)$$

where $\overline{E}$ is the mean content (µg herbicide g^{-1} fresh weight) at 12 h, C is the content of herbicide (µg herbicide g^{-1} fresh weight) at each experimental unit at 24, 72, 168, and 336 HAT. The time needed to reach 50% (H$_{50}$) and 80% (H$_{80}$) herbicide metabolism

in HPPD-R and HPPD-S was determined using the asymmetric three-parameter Weibull model function (W1.3) of the drc package in R statistical software:[35]

$$Y = d \exp \left(- \exp \left(b \left(\log \left(x \right) - e \right) \right) \right) \quad (4)$$

In this model, Y is herbicide (mesotrione or tembotrione) metabolism (%), d is the upper limit, and e represents the inflection point. The parameter b is the relative slope around parameter e, and x is HAT. This was the top model based on Akaike's Information Criteria of the function select in the drc package of R software.

The drc package function *ED* in R software calculated the H$_{50}$ (ED$_{50}$) and H$_{80}$ (ED$_{80}$) herbicide metabolism (%) on HPPD-R and HPPD-S. In addition, the H$_{50}$ ratio indices were compared between P450 inhibitors followed by herbicide and herbicide sprayed alone on HPPD-R. The H$_{50}$ ratio indices were compared using the *EDcomp* function of drc package in R, where P-value < 0.05 indicates that H$_{50}$ are different between treatments.[36]

3 RESULTS

3.1 Dose–response of mesotrione with or without malathion

There was no difference in control of HPPD-R when mesotrione was applied with or without 2000 g a.i. ha^{-1} malathion (Fig. 1). Mesotrione dose providing 50% control of the HPPD-R population was 292 and 241 g a.i. ha^{-1} with and without malathion, respectively (Table 1); however, 80% control was never achieved even with the highest mesotrione rate applied (840 g a.i. ha^{-1}). A similar trend was observed with HPPD-R biomass and density (Fig. S2 and Table S2).

Interestingly, application of malathion followed by 840 g a.i. ha^{-1} mesotrione resulted in up to 70% injury on corn (Figs. 2 and S3). However, the injury was transient, and the effective dose of mesotrione causing 10% injury (ED$_{10}$) increased from 12 g a.i. ha^{-1} at 7 DAT to 283 g a.i. ha^{-1} at 21 DAT, which demonstrated the capacity of corn to metabolize mesotrione even in the presence of
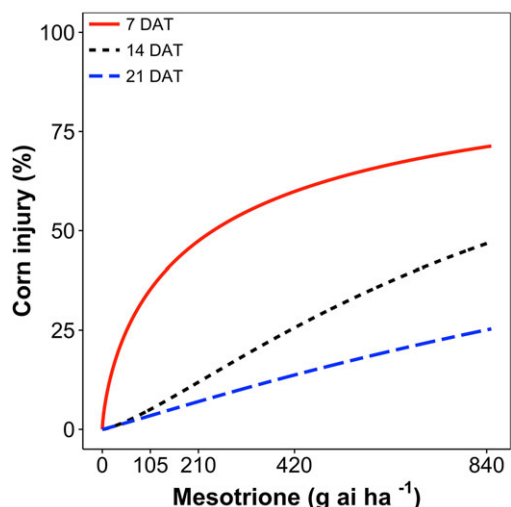
Figure 2. Dose–response of mesotrione with 2000 g a.i. ha^{-1} of malathion on corn injury (%) 7, 14, and 21 days after treatment in a field study in 2014 near Columbus, Platte County, NE, USA.

malathion (Table 2). In addition, the ED$_{50}$ value was reached above the highest mesotrione dose (840 g a.i. ha^{-1}) at 14 and 21 DAT (Table 2). A higher mesotrione dose without malathion caused less than 20% injury at 7 DAT, and no noticeable injury at 14 and 21 DAT (data not shown).

3.2 Efficacy of P450 inhibitors followed bipod-inhibitor for control of HPPD-R population

The effect of P450 inhibitors varied according to the HPPD-inhibitor applied (Table 3). In the greenhouse study, amitrole followed by mesotrione improved control and biomass reduction of HPPD-R by 18% compared with mesotrione applied alone (Table 3). However, malathion followed by mesotrione and PBO followed by mesotrione did not improve efficacy on HPPD-R (Fig. 3). By contrast, malathion, amitrole, and PBO followed by tembotrione and topramezone enhanced HPPD-R control and biomass reduction (Table 3 and Fig. 4). The HPPD-S was sensitive to all treatments applied; all treatment combinations controlled and reduced biomass of HPPD-S ≥ 96% and ≥84%, respectively (Table 3 and Fig. S4).

A similar trend was observed under field conditions (Table 3). For example, HPPD-R control with mesotrione was only 15%, which was not statistically different from malathion followed by

mesotrione and PBO followed by mesotrione (Table 3). Amitrole synergized mesotrione, controlling HPPD-R 58%. The synergistic effect of P450 inhibitors with tembotrione was clearly evident under field conditions. Tembotrione alone controlled HPPD-R 27%, but all cytochrome P450 inhibitors followed by tembotrione provided ≥72% HPPD-R control (Table 3). Similarly, the P450 inhibitors followed by topramezone provided ≥83% HPPD-R control, which was significantly higher than 53% HPPD-R control with topramezone alone. Results on HPPD-R control were corroborated by HPPD-R biomass reduction (Table 3). Therefore, application of malathion, amitrole, or PBO improved the efficacy of tembotrione and topramezone on HPPD-R, whereas only amitrole improved the efficacy of mesotrione on HPPD-R. These results suggest that the mechanism of resistance in this Nebraska HPPD-R population is metabolism-based via increased of P450 activity.

3.3 Influence of P450 inhibitors on mesotrione and tembotrione metabolism

LC–MS/MS analysis of the metabolism of mesotrione and tembotrione is consistent with the herbicidal activity of these herbicides on HPPD-S and HPPD-R in greenhouse and field studies (Fig. 5A). Half of the mesotrione absorbed in HPPD-R remained after 19 h (H$_{50}$) when applied alone, or following malathion and PBO (Tables 4 and S3). However, amitrole synergized mesotrione and the H$_{50}$ was reached later at 28 HAT. Amitrole followed by mesotrione delayed mesotrione metabolism by 50% in comparison with mesotrione alone on HPPD-R (1.5-fold) (Table 4). A similar trend was observed for 80% mesotrione metabolism (H$_{80}$) on HPPD-R. For example, 80% of mesotrione was metabolized by HPPD-R applied alone or with malathion or PBO in less than 25 HAT. Again, amitrole slowed the rate of herbicide metabolism, requiring 48 h to reach 80% mesotrione metabolism (Table 4). Mesotrione alone on HPPD-S was included as a positive control; the time required to reach 50 and 80% mesotrione metabolism was 33 and 79 HAT, respectively. The time for mesotrione metabolism in the presence of amitrole falls in between mesotrione alone in HPPD-R and HPPD-S (Table 4), demonstrating a moderate increase in efficacy of HPPD-inhibitor on HPPD-R when amitrole is used.

LC–MS/MS analysis of tembotrione metabolism was also consistent with the efficacy of this herbicide in greenhouse and field studies on HPPD-S and HPPD-R biotypes (Fig. 5B). Malathion, amitrole, and PBO synergized tembotrione. For example, 50% of tembotrione was metabolized 19 HAT on HPPD-R (Tables 5

Table 2. Estimated parameters and ED$_{10}$, ED$_{30}$, and ED$_{50}$ from the dose–response of mesotrione with 2000 g a.i. ha^{-1} of malathion on corn injury (%) 7, 14, and 21 days after treatment in a field study in 2014 near Columbus, Platte County, NE, USA.

				Parameter[b]		
	ED$_{10}$ (± SE)[a]	ED$_{30}$ (± SE)[a]	ED$_{50}$ (± SE)[a]	b (± SE)	d (± SE)	e (ED50) (± SE)
DAT		(g a.i. ha^{-1})			(%)	(g a.i. ha^{-1})
7	12 (7)	75 (20)	242 (32)	-0.7 (0.1)	100	242 (32)
14	182 (37)	494 (48)	925 (121)	-1.4 (0.2)	100	925 (121)
21	283 (62)	1054 (202)	2320 (880)	-1.1 (0.3)	100	2320 (880)

DAT, days after treatment application.
[a] ED$_{10}$, mesotrione dose needed to cause 10% injury on corn; ED$_{30}$, mesotrione dose needed to cause 30% injury on corn; ED$_{50}$, mesotrione dose needed to cause 50% injury on corn; SE, standard error.
[b] b, the slope; d, the upper limit (locked at 100); and e, the inflection point relative to the upper limit.

Table 3. Effect of P450 inhibitors followed by (fb) mesotrione, tembotrione, and topramezone on HPPD-inhibitor resistant (HPPD-R) and susceptible (HPPD-S)-*A. tuberculatus* control (%), biomass reduction (%), and % corn injury (field only) in a greenhouse in 2015 at the University of Nebraska-Lincoln and a field experiment in 2016 near Columbus, Platte County, NE, USA

	Greenhouse				Field		
	Control[a]	Biomass reduction[a]	Control	Biomass reduction	Control	Biomass reduction	Injury
	A. tuberculatus biotype[b] (%)						
Treatment	HPPD-S		HPPD-R		HPPD-R		Corn
Untreated	–	–	–	–	–	–	–
Mesotrione	96 a	84 a	57 b	56 b	15 b	11 b	0
Malathion fb mesotrione	97 a	85 a	55 b	63 ab	20 b	13 b	1
Amitrole fb mesotrione	97 a	86 a	75 a	74 a	58 a	70 a	1
PBO fb mesotrione	96 a	85 a	68 b	71 ab	28 b	22 b	2
Tembotrione	98 a	86 a	75 b	70 b	27 c	40 c	1
Malathion fb tembotrione	98 a	89 a	91 a	83 a	84 a	91 a	1
Amitrole fb tembotrione	98 a	87 a	89 a	83 a	72 ab	76 b	1
PBO fb tembotrione	98 a	87 a	93 a	81 a	81 a	91 a	1
Topramezone	98 a	89 a	82 b	73 b	53 b	77 b	1
Malathion fb topramezone	98 a	88 a	89 a	88 a	86 a	92 a	1
Amitrole fb topramezone	97 a	86 a	90 a	87 a	83 a	93 a	1
PBO fb topramezone	97 a	84 a	92 a	89 a	84 a	90 a	1
P-value[c]	0.71	0.77	<0.01	<0.01	<0.01	<0.01	-

[a] Means presented within each column with no common letter(s) are significantly different according to Fisher's Protected LSD test where *P* < 0.05. Results are presented separately for each herbicide.
[b] HPPD-S, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor herbicide-susceptible *A. tuberculatus* collected from a field in Dixon County, NE in 2014. HPPD-R, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor herbicide-resistant *A. tuberculatus* collected from a field in Platte County, NE in 2014.
[c] ANOVA, *P* < 0.05 indicates a significant difference among treatments.
PBO, piperonyl butoxide.
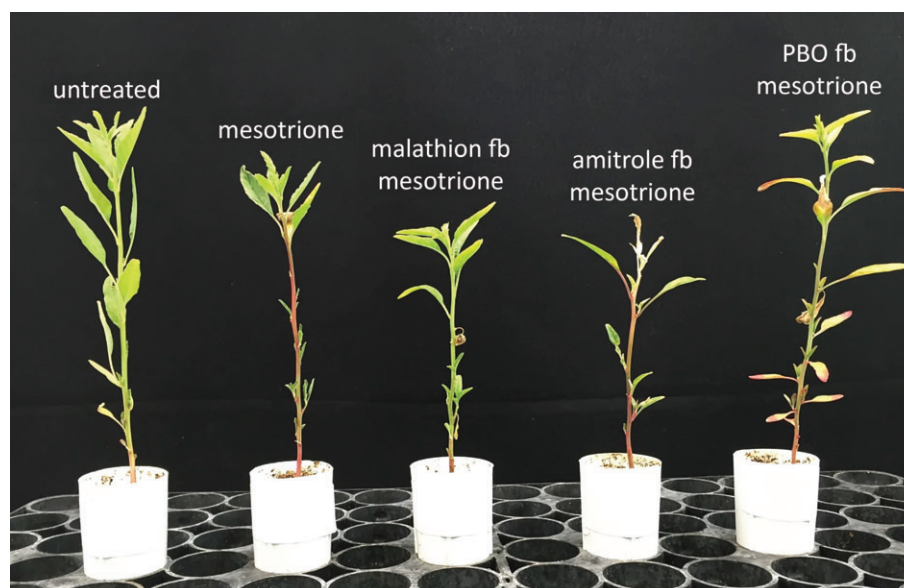
Figure 3. Efficacy of cytochrome P450 inhibitors [malathion, amitrole, and piperonyl butoxide (PBO)] followed by (fb) mesotrione in the HPPD-inhibitor herbicide resistant *Amaranthus tuberculatus* 21 days after treatment in a greenhouse study in 2015 at the University of Nebraska-Lincoln.
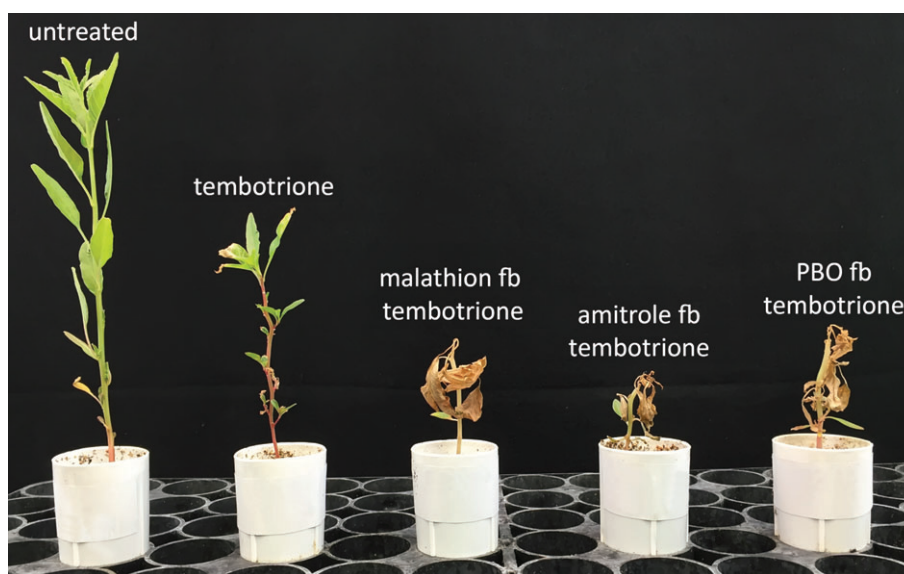
Figure 4. Efficacy of cytochrome P450 inhibitor [malathion, amitrole, and piperonyl butoxide (PBO)] followed by (fb) tembotrione in the HPPD-inhibitor herbicide resistant *Amaranthus tuberculatus* 21 days after treatment in a greenhouse study in 2015 at the University of Nebraska-Lincoln.
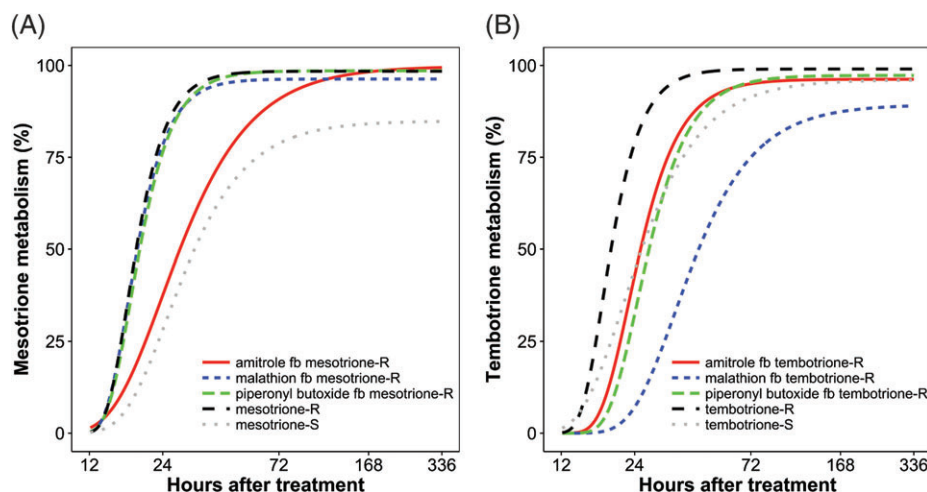
Figure 5. Mesotrione (A) and tembotrione (B) metabolism (%) in HPPD-R and HPPD-S from 12 to 336 h after treatment. Treatments with -R were applied on HPPD-R, whereas treatments with -S were applied on HPPD-S.

and S4), whereas it required more than 26 h to achieve a similar level of metabolism when cytochrome P450 inhibitors were used. Malathion provided the highest level of synergistic effect on tembotrione (Table 5). The H_{50} in malathion followed by tembotrione was 2.2-fold the H_{50} of tembotrione alone on HPPD-R. Moreover, the time needed to reach 80% tembotrione metabolism on HPPD-R was $\geq$36 HAT when cytochrome P450 inhibitors followed by tembotrione were sprayed. In the HPPD-S, the H_{50} and H_{80} were 26 and 43 HAT, respectively (Table 5). The smaller difference between HPPD-R and HPPD-S tembotrione metabolism is likely due to the moderate resistance level to tembotrione (6×), as demonstrated in previous research.[33] Nonetheless, there was a strong synergistic effect of P450 inhibitors on the efficacy of tembotrione on the HPPD-R population. The times for H_{50} and H_{80} of tembotrione on HPPD-S and cytochrome P450 followed by tembotrione on HPPD-R are similar. As a result, P450 inhibitors followed by tembotrione reversed the HPPD-R to a susceptible phenotype.

4 DISCUSSION

Amaranthus tuberculatus resistance to HPPD inhibitors has primarily evolved through the selection of NTSR mechanisms. Enhanced mesotrione metabolism was previously reported in an *A. tuberculatus* biotype from Illinois, in which Ma *et al.*[25] reported that malathion does synergize mesotrione, increasing *A. tuberculatus* control. However, in HPPD-R from Nebraska, malathion did not synergize mesotrione (Fig. 1). Therefore, it was hypothesized that the P450 gene(s) causing mesotrione resistance in the Nebraska HPPD-R population are different from the gene(s) responsible for resistance in the Illinois population, due to the observed differences in inhibition by malathion. This evidence suggests that multiple, different P450 genes appear to have evolved for mesotrione resistance, and they are different between the *A. tuberculatus* populations.

The cytochrome P450 family is one of the largest gene families in plants, with over 300 genes.[37] Although the organophosphate insecticide malathion, the synergist chemical PBO, and herbicide

Table 4. Estimated H_{50} and H_{80} values based on mesotrione metabolism (%) in 8–10 cm tall HPPD-R and HPPD-S 21 days after application of cytochrome P450 inhibitors fb mesotrione.

Treatment	Biotype	H_{50} ($\pm$SE)[a]	H_{80} ($\pm$SE)[a]	P-value[b]	Ratio[c]
Mesotrione	HPPD-S	33 (2)	79 (15)	–	–
Mesotrione	HPPD-R	19 (2)	24 (1)	–	–
Malathion fb mesotrione	HPPD-R	19 (1)	25 (1)	0.90	1.0
Amitrole fb mesotrione	HPPD-R	28 (1)	48 (4)	<0.01	1.5
PBO fb mesotrione	HPPD-R	19 (1)	25 (1)	0.77	1.0

HPPD-S, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor herbicide-susceptible *A. tuberculatus* collected from a field in Dixon County, NE in 2014. HPPD-R, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor herbicide-resistant *A. tuberculatus* collected from a field in Platte County, NE in 2014. PBO, piperonyl butoxide.
[a] H_{50}, hours after treatment application needed to reach 50% mesotrione metabolism on HPPD-S and HPPD-R; H_{80}, hours after treatment application needed to reach 80% mesotrione metabolism on HPPD-S and HPPD-R
[b] Mesotrione on HPPD-R vs. cytochrome P450 inhibitors fb mesotrione treatments on HPPD-R t-statistics comparison of H_{50}. $P < 0.05$ indicates a significant difference between treatments.
[c] Time ratio indices were calculated by dividing the H_{50} value of P450 inhibitors followed by (fb) mesotrione on HPPD-R treatments by mesotrione on HPPD-R.

Table 5. Estimated H_{50} and H_{80} values based on tembotrione metabolism (%) in 8–10 cm tall HPPD-R and HPPD-S 21 days after application of cytochrome P450 inhibitors fb tembotrione

Treatment	Biotype	H_{50} ($\pm$ SE)[a]	H_{80} ($\pm$ SE)[a]	P-value[b]	Ratio[c]
Tembotrione	HPPD-S	26 (1)	43 (4)	–	–
Tembotrione	HPPD-R	19 (3)	24 (1)	–	–
Malathion fb tembotrione	HPPD-R	43 (2)	90 (10)	<0.01	2.2
Amitrole fb tembotrione	HPPD-R	26 (1)	36 (7)	<0.01	1.4
PBO fb tembotrione	HPPD-R	28 (1)	38 (6)	<0.01	1.4

HPPD-S, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor herbicide-susceptible *A. tuberculatus* collected from a field in Dixon County, NE in 2014. HPPD-R, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor herbicide-resistant *A. tuberculatus* collected from a field in Platte County, NE in 2014. PBO, piperonyl butoxide.
[a] H_{50}, hours after treatment application needed to reach 50% tembotrione metabolism on HPPD-S and HPPD-R; H_{80}, hours after treatment application needed to reach 80% tembotrione metabolism on HPPD-S and HPPD-R
[b] Tembotrione on HPPD-R vs. cytochrome P450 inhibitors fb tembotrione treatments on HPPD-R t-statistics comparison of H_{50}. $P < 0.05$ indicates a significant difference between treatments.
[c] Time ratio indices were calculated by dividing the H_{50} value of P450 inhibitors followed by (fb) tembotrione on HPPD-R treatments by tembotrione on HPPD-R.

amitrole inhibit plant P450,[38] each appears to target different classes of P450. For example, amitrole is a herbicide with an unknown mechanism of resistance, causing bleaching in new plant tissue.[39] Amitrole was reported to revert diclofop-methyl resistance in a *Lolium rigidum* phenotype, but not chlorsulfuron resistance.[40] Also, malathion reverses only chlorsulfuron resistance and amitrole reverses only diclofop resistance in different *L. rigidum* phenotypes.[29] Thus, the complexity of P450 enzymes warrants further investigation on different P450 inhibitors in resistant plant phenotypes.

The HPPD-R control was enhanced with amitrole followed by mesotrione application but never reached near 90% in greenhouse and field studies (Table 3). By contrast, malathion and PBO followed by mesotrione did not reverse resistance to this herbicide. Resistance to tembotrione and topramezone appears to involve a different set of P450s than those imparting resistance to mesotrione because the application of malathion, amitrole, or PBO reversed the resistance to these two herbicides in HPPD-R (Table 3). Therefore, our hypothesis is accepted for tembotrione and topramezone but rejected for mesotrione (except for amitrole). Tembotrione was metabolized quickly in HPPD-R leaves when applied alone, whereas its rate of metabolism was reduced when malathion, amitrole, and PBO were applied prior to

tembotrione (Fig. 5B). This reduction in tembotrione metabolism was sufficient to restore herbicidal activity on HPPD-R. The different patterns of reversal achieved with malathion, PBO, and amitrole on mesotrione and tembotrione suggest that multiple P450 genes are involved in metabolism-based resistance to these structurally similar herbicides.

Mesotrione resistance in HPPD-R is due to detoxification of parent compound into 4-hydroxymesotrione, and it is not associated with TSR mechanisms.[34] HPPD resistance in *A. palmeri* from Kansas was shown to involve enhanced metabolism of mesotrione[23] and tembotrione,[24] along with the potential contribution of increased HPPD expression.[23] Our results further characterized a role for multiple P450 traits in enhanced mesotrione and tembotrione metabolism in HPPD-R. It is likely that selection for resistance in HPPD-R was fostered by low herbicide rates, poor timing, and suboptimal herbicide application conditions. Plants were able to survive by rapid herbicide metabolism, transferring resistance genes to the next generation through cross-pollination and thereby spreading moderately high resistance levels and accumulating multiple P450 alleles contributing to HPPD resistance. An inheritance study in HPPD-inhibitor resistant *A. tuberculatus* from Illinois suggested that resistance was polygenic.[41] Although the mode of inheritance in HPPD-R remains unknown, the specificity

of P450 and herbicide interactions to reverse resistance indicate that multiple P450 alleles are conferring resistance in HPPD-R.

This study highlights the complexity of NTSR mechanisms involving P450. The large number of P450s, each with its own substrate specificity, combined with the high genetic diversity present in obligate out-crosser species make the metabolism-based resistance in *A. tuberculatus* a serious concern to corn and soybean producers in the north–central USA. In the near future, HPPD-inhibitor resistant soybean and cotton will be commercialized, and this is likely to increase the selection pressure of HPPD-inhibitors. Also, P450 can confer unpredictable cross-resistance to other herbicides,[15] which can reduce the value of herbicide mixtures for delaying resistance evolution. Moreover, no new herbicide mode of action is expected to appear in the near future.[42] Therefore, NTSR will make weed management incrementally more difficult. The use of synergists may be a part of future solutions and it opens a research field which needs further exploration. Studies have demonstrated the capacity of synergists to revert resistance.[43–45] Major concerns with synergists are that these molecules may also reduce crop selectivity and may have an unintended environmental impact.[46–49] Nonetheless, the organophosphate insecticide phorate (a P450 inhibitor) provided a high level of crop safety against injury by clomazone and triallate to rice seedlings.[50] In this study, corn injury was either low or transient and the crop recovered within 21 DAT. Also, malathion, amitrole, and PBO followed by tembotrione reversed HPPD-R to a susceptible phenotype. Thus, these synergists might be useful tools in combatting metabolism-based herbicide resistance as a part of new stewardship management programs.

5 CONCLUSION

This study confirms the enhanced metabolism-based mesotrione, tembotrione, and topramezone resistance via P450 enzymes in HPPD-R. It was demonstrated that multiple P450 enzymes are causing resistance in HPPD-R. It remains unidentified whether another NTSR mechanism has arisen in this population. Post-emergence application of P450 inhibitors, including malathion, amitrole, and PBO with HPPD-inhibitor herbicides (mesotrione, tembotrione, and topramezone) showed a potential for reversing HPPD-R to a susceptible phenotype. However, fully elucidated weed management strategies will require additional investigation on candidate P450 alleles causing this striking resistance.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Johnson WG, Davis VM, Kruger GR and Weller SC, Influence of glyphosate-resistant cropping systems on weed species shifts and glyphosate-resistant weed populations. *Eur J Agron* **31**:162–172 (2009).

2 Culpepper AS, Glyphosate-induced weed shifts. *Weed Technol* **20**:277–281 (2006).

3 Refsell DE and Hartzler RG, Effect of tillage on common waterhemp (*Amaranthus rudis*) emergence and vertical distribution of seed in the soil. *Weed Technol* **23**:129–133 (2009).

4 Owen MDK, Weed species shifts in glyphosate-resistant crops. *Pest Manag Sci* **64**:377–387 (2008).

5 Sarangi D, Irmak S, Lindquist JL, Knezevic SZ and Jhala AJ, Effect of water stress on the growth and fecundity of common waterhemp (*Amaranthus rudis*). *Weed Sci* **64**:42–52 (2016).

6 Costea M, Weaver SE and Tardif FJ, The biology of invasive alien plants in Canada. 3. *Amaranthus tuberculatus* (Moq.) Sauer var. *rudis* (Sauer) Costea & Tardif. *Can J Plant Sci* **85**:507–522 (2005).

7 Wu C and Owen MDK, When is the best time to emerge: reproductive phenology and success of natural common waterhemp (*Amaranthus rudis*) cohorts in the midwest United States? *Weed Sci* **62**:107–117 (2014).

8 Bensch CN, Horak MJ and Peterson D, Interference of redroot pigweed (*Amaranthus retroflexus*), Palmer amaranth (*A. palmeri*), and common waterhemp (*A. rudis*) in soybean. *Weed Sci* **51**:37–43 (2003).

9 Hartzler RG, Battles BA and Nordby D, Effect of common waterhemp (*Amaranthus rudis*) emergence date on growth and fecundity in soybean. *Weed Sci* **52**:242–245 (2004).

10 Franssen AS, Skinner DZ, Al-Khatib K, Horak MJ and Kulakow PA, Interspecific hybridization and gene flow of ALS resistance in *Amaranthus* species. *Weed Sci* **49**:598–606 (2001).

11 Gaines TA, Ward SM, Bukun B, Preston C, Leach JE, Westra P, Interspecific hybridization transfers a previously unknown glyphosate resistance mechanism in Amaranthus species. *Evol Appl* **5**:29–38 (2012).

12 Sarangi D, Tyre AJ, Patterson EL, Gaines TA, Irmak S, Knezevic SZ *et al.*, Pollen-mediated gene flow from glyphosate-resistant common waterhemp (*Amaranthus rudis* Sauer): consequences for the dispersal of resistance genes. *Sci Rep* **7**:44913 (2017).

13 Heap I, *The International Survey of Herbicide Resistant Weeds*. Available from www.weedscience.org [accessed 10 July 2017].

14 Beckie HJ and Tardif FJ, Herbicide cross resistance in weeds. *Crop Protect* **35**:15–28 (2012).

15 Délye C, Jasieniuk M and Le Corre V, Deciphering the evolution of herbicide resistance in weeds. *Trends Genet* **29**:649–658 (2013).

16 Chatham LA, Bradley KW, Kruger GR, Martin JR, Owen MDK, Peterson DE *et al.*, A multistate study of the association between glyphosate resistance and EPSPS gene amplification in waterhemp (*Amaranthus tuberculatus*). *Weed Sci* **63**:569–577 (2015).

17 Chatham LA, Wu C, Riggins CW, Hager AG, Young BG, Roskamp GK *et al.*, EPSPS gene amplification is present in the majority of glyphosate-resistant Illinois waterhemp (*Amaranthus tuberculatus*) populations. *Weed Technol* **29**:48–55 (2015).

18 Patzoldt WL, Hager AG, McCormick JS and Tranel PJ, A codon deletion confers resistance to herbicides inhibiting protoporphyrinogen oxidase. *Proc Natl Acad Sci USA* **103**:12329–12334 (2006).

19 Powles SB and Yu Q, Evolution in action: plants resistant to herbicides. *Ann Rev Plant Biol* **61**:317–347 (2010).

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

21 Hausman NE, Singh S, Tranel PJ, Riechers DE, Kaundun SS, Polge ND *et al.*, Resistance to HPPD-inhibiting herbicides in a population of waterhemp (*Amaranthus tuberculatus*) from Illinois, United States. *Pest Manag Sci* **67**:258–261 (2011).

22 McMullan PM and Green JM, Identification of a tall waterhemp (*Amaranthus tuberculatus*) biotype resistant to HPPD-inhibiting herbicides, atrazine, and thifensulfuron in Iowa. *Weed Technol* **25**:514–518 (2011).

23 Nakka S, Godar AS, Wani PS, Thompson CR, Peterson DE, Roelofs J *et al.*, Physiological and molecular characterization of hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor resistance in Palmer amaranth (*Amaranthus palmeri* S. Wats.). *Front Plant Sci* **8**:555 (2017).

24 Küpper A, Peter F, Zöllner P, Lorentz L, Tranel PJ, Beffa R *et al.*, Tembotrione detoxification in HPPD-inhibitor resistant Palmer amaranth (Amaranthus palmeri S. Wats.). *Pest Manage Sci* (2017).

25 Ma R, Kaundun SS, Tranel PJ, Riggins CW, McGinness DL, Hager AG *et al.*, Distinct detoxification mechanisms confer resistance to mesotrione and atrazine in a population of waterhemp. *Plant Physiol* **163**:363–377 (2013).

26 Beckie HJ, Herbicide-resistant weeds: management tactics and practices. *Weed Technol* **20**:793–814 (2006).

27 Norsworthy JK, Ward SM, Shaw DR, Llewellyn RS, Nichols RL, Webster TM et al., Reducing the risks of herbicide resistance: best management practices and recommendations. Weed Sci **60**:31–62 (2012).

28 Oliveira MC, Feist D, Eskelsen S, Scott JE and Knezevic SZ, Weed control in soybean with preemergence- and postemergence-applied herbicides. Crop Forage Turfgrass Manag **3**:1–7 (2017).

29 Yu Q and Powles SB, Metabolism-based herbicide resistance and cross-resistance in crop weeds: a threat to herbicide sustainability and global crop production. Plant Physiol **166**:1106–1118 (2014).

30 Yu Q, Abdallah I, Han HP, Owen M and Powles S, Distinct non-target site mechanisms endow resistance to glyphosate, ACCase and ALS-inhibiting herbicides in multiple herbicide-resistant Lolium rigidum. Planta **230**:713–723 (2009).

31 Cummins I, Wortley DJ, Sabbadin F, He Z, Coxon CR, Straker HE et al., Key role for a glutathione transferase in multiple-herbicide resistance in grass weeds. Proc Natl Acad Sci USA **110**:5812–5817 (2013).

32 Christopher JT, Preston C and Powles SB, Malathion antagonizes metabolism-based chlorsulfuron resistance in Lolium rigidum. Pestic Biochem Physiol **49**:172–182 (1994).

33 Oliveira MC, Jhala AJ, Gaines T, Irmak S, Amundsen K, Scott JE et al., Confirmation and control of HPPD-inhibiting herbicide–resistant waterhemp (Amaranthus tuberculatus) in Nebraska. Weed Technol **31**:67–79 (2017).

34 Kaundun SS, Hutchings S-J, Dale RP, Howell A, Morris JA, Kramer VC et al. Mechanism of resistance to mesotrione in an Amaranthus tuberculatus population from Nebraska, USA. PLoS ONE **12**:e0180095 (2017).

35 Ritz C, Baty F, Streibig JC and Gerhard D, Dose–response analysis using R. PLoS ONE **10**:e0146021 (2016).

36 Ritz C and Streibig JC, Bioassay analysis using R. J Stat Software **12**:1–22 (2005).

37 Werck-Reichhart D and Feyereisen R, Cytochromes P450: a success story. Genome Biol 1:reviews3003.1 (2000).

38 Preston C, Herbicide resistance in weeds endowed by enhanced detoxification: complications for management. Weed Sci **52**:448–453 (2004).

39 Hilton JL, Inhibitions of growth and metabolism by 3-amino-1,2,4-triazole (amitrole). J Agric Food Chem **17**:182–198 (1969).

40 Preston C and Powles SB, Amitrole inhibits diclofop metabolism and synergises diclofop-methyl in a diclofop-methyl-resistant biotype of Lolium rigidum. Pestic Biochem Physiol **62**:179–189 (1998).

41 Huffman J, Hausman NE, Hager AG, Riechers DE and Tranel PJ, Genetics and inheritance of nontarget-site resistances to atrazine and mesotrione in a waterhemp (Amaranthus tuberculatus) population from Illinois. Weed Sci **63**:799–809 (2015).

42 Duke SO, Why have no new herbicide modes of action appeared in recent years? Pest Manag Sci **68**:505–512 (2012).

43 Preston C, Tardif FJ, Christopher JT and Powles SB, Multiple resistance to dissimilar herbicide chemistries in a biotype of Lolium rigidum due to enhanced activity of several herbicide degrading enzymes. Pestic Biochem Physiol **54**:123–134 (1996).

44 Burnet MWM, Loveys BR, Holtum JAM and Powles SB, A mechanism of chlorotoluron resistance in Lolium rigidum. Planta **190**:182–189 (1993).

45 Busi R, Gaines TA and Powles S, Phorate can reverse P450 metabolism-based herbicide resistance in Lolium rigidum. Pest Manag Sci **73**:410–417 (2017).

46 Diehl KE, Stoller EW and Barrett M, In vivo and in vitro inhibition of nicosulfuron metabolism by terbufos metabolites in maize. Pestic Biochem Physiol **51**:137–149 (1995).

47 Barrett M, Metabolism of herbicides by cytochrome P450 in corn. Drug Metab Drug Interact **12**:299–316 (1995).

48 Nordby JN, Williams MM, Pataky JK, Riechers DE and Lutz JD, A common genetic basis in sweet corn inbred Cr1 for cross sensitivity to multiple cytochrome P450-metabolized herbicides. Weed Sci **56**:376–382 (2008).

49 Steckel LE, Stewart SD and Steckel S, Corn response to POST-applied HPPD-inhibitor based premix herbicides with in-furrow and foliar-applied insecticides. Weed Technol **29**:18–23 (2015).

50 Busi R, Nguyen NK, Chauhan BS, Vidotto F, Tabacchi M and Powles SB, Can herbicide safeners allow selective control of weedy rice infesting rice crops? Pest Manag Sci **73**:71–77 (2017).