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Resistance to 2,4-D in Palmer amaranth (*Amaranthus palmeri*) from Kansas is mediated by enhanced metabolism

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Abstract

A Palmer amaranth (*Amaranthus palmeri* S. Watson) population (KCTR: KS Conservation Tillage Resistant) collected from a conservation tillage field was confirmed with resistance to herbicides targeting at least six sites of action, including 2,4-D. The objectives of this research were using KCTR *A. palmeri* to investigate (1) the level of 2,4-D resistance, (2) 2,4-D absorption and translocation profiles, (3) the rate of 2,4-D metabolism compared with 2,4-D–tolerant wheat (*Triticum aestivum* L.), and (4) the possible role of cytochrome P450s (P450s) in mediating resistance. Dose–response experiments were conducted to assess the level of 2,4-D resistance in KCTR compared with susceptible plants, KSS (KS 2,4-D susceptible) and MSS (MS 2,4-D susceptible). KSS, MSS, and KCTR plants were treated with [^{14}C]2,4-D to determine absorption, translocation, and metabolic patterns. Additionally, whole-plant dose–response assays were conducted by treating KCTR and KSS plants with P450 inhibitors (malathion, piperonyl butoxide [PBO]) before 2,4-D application. Dose–response experiments indicated a 6- to 11-fold 2,4-D resistance in KCTR compared with susceptible plants. No difference was found in percent [^{14}C]2,4-D absorption among the populations. However, 10% less and 3 times slower translocation of [^{14}C]2,4-D was found in KCTR compared with susceptible plants. Importantly, [^{14}C]2,4-D was metabolized faster in KCTR than susceptible plants. At 24, 48, and 72 h after treatment (HAT), KCTR metabolized ~20% to 30% more [^{14}C]2,4-D than susceptible plants. KCTR plants and wheat generated metabolites with similar polarity. Nonetheless, at 24 HAT, ~70% of [^{14}C]2,4-D was metabolized in wheat, compared with only 30% in KCTR *A. palmeri*. Application of malathion before 2,4-D increased the sensitivity to 2,4-D in KCTR, suggesting involvement of P450s in mediating 2,4-D metabolism. However, no such impact of PBO was documented. Overall, this study confirms that enhanced metabolism is the primary mechanism of 2,4-D resistance in KCTR.

Introduction

The synthetic auxin herbicide (SAH) 2,4-D has been used to control broadleaf weeds since 1945 (Peterson et al. 2016). Conventionally, SAHs such as 2,4-D are widely used to control broadleaf weeds in cereals crops, such as sorghum [*Sorghum bicolor* (L.) Moench], wheat (*Triticum aestivum* L.), and corn (*Zea mays* L.). However, over the years, SAHs (viz., quinclorac and halauxifen-methyl) were found to have the ability to control certain grass weeds as well. Despite extensive use of SAHs (e.g., 2,4-D), the evolution of resistance to these herbicides is relatively slow compared with other herbicide groups such as triazines or acetolactate synthase (ALS) inhibitors (Busi et al. 2017). However, in the past two decades, increased reliance on and usage of 2,4-D for controlling weeds resistant to ALS and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitors have led to a rapid increase in resistance to 2,4-D in weeds. Currently, 2,4-D resistance is documented in 41 weeds globally (Heap 2022). Moreover, 2,4-D–tolerant crops, that is, soybean [*Glycine max* (L.) Merr.], corn, and cotton (*Gossypium hirsutum* L.), were recently commercialized, which can aid in controlling troublesome weeds such as glyphosate-resistant Palmer amaranth (*Amaranthus palmeri* S. Watson) (Shyam et al. 2020). Intensified adoption of this technology is also expected to cause a surge in 2,4-D use, resulting in increased selection pressure and potentially leading to 2,4-D resistance evolution (Egan et al. 2011). This scenario calls for a thorough characterization and investigation of prevalent mechanisms bestowing 2,4-D resistance in weeds, which in turn can help formulate sustainable weed management programs and increase the longevity of 2,4-D-resistant crop technology.

Resistance to 2,4-D in weeds is reported to be conferred either by target-site resistance (TSR)- or non–target site resistance (NTSR)-based mechanisms. In 2018, the first confirmed case of TSR was documented in a kochia [*Bassia scoparia* (L.) A.J. Scott] population from Nebraska, where a mutation in the auxin receptor protein KS18A resulted in dicamba resistance

(LeClere et al. 2018). More recently, a case of a TSR mechanism with a 20 amino acid deletion in the auxin co-receptor *IAA2* was reported to cause 2,4-D resistance in an Indian hedge mustard population (*Sisymbrium orientale* L.) from Australia (Figueiredo et al. 2021). In contrast, a multitude of weeds were documented with NTSR involving either reduced absorption (Kohler et al. 2004), translocation (Dang et al. 2018; Kohler et al. 2004; Riar et al. 2011), and/or enhanced metabolism (Figueiredo et al. 2018; Torra et al. 2017) of SAHs. For instance, reduced 2,4-D translocation was reported in *S. orientale* (Dang et al. 2018), ground ivy (*Glechoma hederacea* L.) (Kohler et al. 2004), prickly lettuce (*Lactuca serriola* L.) (Riar et al. 2011), wild radish (*Raphanus raphanistrum* L.) (Goggin et al. 2016), and corn poppy (*Papaver rhoeas* L.) (Rey-Caballero et al. 2016). Enhanced metabolism of 2,4-D was also documented in waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] populations across the U.S. Midwest (Figueiredo et al. 2018; Shergill et al. 2018; Shyam et al. 2019) and *P. rhoeas* populations in Spain (Torra et al. 2017). Likewise, natural tolerance to 2,4-D in grasses is often attributed to the rapid metabolism of this herbicide compared with susceptible broadleaved weeds. Metabolism of 2,4-D is well characterized in naturally tolerant cereal crops such as corn (Fang and Butts 1954), wheat (Bristol et al. 1977; Fang and Butts 1954; Hamburg et al. 2001; Scheel and Sandermann 1981), and sorghum (Morgan and Hall 1963). Cytochrome P450 monooxygenases (P450s) are known to metabolize herbicides, including SAHs, imparting selectivity in tolerant crops (Frear 1995; McFadden et al. 1989; Xiang et al. 2006). P450s are one of the major families of enzymes involved in Phase I herbicide metabolism, thus offering herbicide selectivity to some plants (Pandian et al. 2020).

Amaranthus palmeri, one of the top-ranked troublesome weeds in the United States (Van Wychen 2020), is a C_4 plant that can accumulate biomass rapidly and has a high growth rate compared with other *Amaranthus* spp. (Horak and Loughin 2000; Wang et al. 1992). Being a dioecious plant, *A. palmeri* is an obligate outcrosser and possesses high genetic diversity. This allows the weed to adapt quickly to challenging abiotic and biotic stress conditions and it's the ability to evolve resistance to herbicides. So far, *A. palmeri* has evolved resistance to herbicides targeting nine sites of action (SOAs) (Heap 2022).

A KCTR (KS Conservation Tillage Resistant) *A. palmeri* population was found to be resistant to multiple herbicides, including ALS, photosystem II (PSII), EPSPS, protoporphyrinogen oxidase, 4-hydroxphenylpyruvate dioxygenase (HPPD) inhibitors and SAH (2,4-D) (Shyam et al. 2021). Eighty-four percent of KCTR plants survived 2,4-D treatment at the field-recommended dose of 560 g ae ha^{-1}, confirming the evolution of resistance (Shyam et al. 2021). In addition to the KCTR population, evolution of 2,4-D resistance has been reported in another *A. palmeri* population in Kansas (Kumar et al. 2019). Nonetheless, information on the mechanism of 2,4-D resistance in *A. palmeri* is lacking. Therefore, the objectives of this research were to investigate KCTR for (1) the level of 2,4-D resistance, (2) 2,4-D absorption and translocation profiles, (3) the rate of 2,4-D metabolism compared with 2,4-D–tolerant wheat, and (4) the possible role of P450s in mediating resistance.

Materials and Methods

Plant Material and Growing Conditions

Amaranthus palmeri 2,4-D–resistant KCTR and susceptible KSS (KS susceptible) and MSS (MS susceptible) populations were used in this research. The KCTR population was collected from a conservation tillage field and was characterized previously (Shyam et al. 2021). The KSS population was collected from a nearby field at Ashland Bottoms, KS, and is susceptible to herbicides commonly used to control *A. palmeri* (Nakka et al. 2017b, 2017a, 2017c; Shyam et al. 2021). Seeds of the MSS population, which is susceptible to common herbicides used to control *A. palmeri* (Nakka et al. 2017b, 2017a, 2017c; Shyam et al. 2021), were purchased from a vendor (Azlin Seed Services, Leland, MS). Experiments were conducted in either greenhouse or controlled environment growth-chamber conditions. The greenhouse was maintained at 30/23 C day/night (d/n) temperatures with a 16/8-h photoperiod (d/n) and 60 ± 10% relative humidity. Natural light in the greenhouse was supplemented with 250 μmol m^{-2} s^{-1} illumination provided by sodium vapor lamps. Growth chambers were maintained at 32.5/22.5 C (d/n) temperatures with a photoperiod of 15/9 h (d/n) and relative humidity of 60 ± 10%. Lighting intensity of 750 μmol m^{-2} s^{-1} was provided inside the growth chambers through fluorescent and incandescent bulbs. KCTR, KSS, and MSS seedlings were germinated in trays (21 by 6 by 4 cm) filled with a commercial pre-mix (Pro-Mix® premium potting mix, Premier Tech Home and Garden, Mississauga, ON, Canada). Three- or four-leaf seedlings were transplanted to individual pots (6 by 6 by 6.5 cm) and allowed to grow in either the greenhouse or growth chambers.

Dose–Response with 2,4-D

Whole-plant 2,4-D dose–response experiments were conducted to characterize the level of 2,4-D resistance in KCTR compared with the susceptible KSS and MSS populations. These experiments were performed in a completely randomized design in growth chambers, and they were repeated. For the experiment, KCTR, KSS, and MSS seedlings were germinated in trays (21 by 6 by 4 cm) under greenhouse conditions and transplanted into small pots (6 by 6 by 6.5 cm). After transplanting, the seedlings were transferred to growth chambers. Seedlings were treated at the 10- to 12-cm stage with 2,4-D (2,4-D 4L Amine, Winfield Solutions, St Paul, MN) at the following doses: 0, 140, 280, 560 (field recommended dose), 1,120, 2,240, 4,480, 8,690 g ae ha^{-1}. Seedlings were returned to the growth chambers within 30 min of treatment. The 2,4-D applications were done using a bench-track sprayer (Generation III, DeVries Manufacturing, Hollandale, MN) equipped with a flat-fan nozzle tip (TeeJet® 8002, Spraying Systems, Wheaton, IL). The sprayer was calibrated to deliver a spray volume proportional to 187 L ha^{-1} at a speed of 4.77 km h^{-1}. Five replications were maintained for each treatment, and the experiment was repeated once. Four weeks after treatment (WAT), aboveground biomass was harvested from these seedlings, oven-dried, and weighed. For data analysis, the dry weight data were converted to relative dry weight (% of nontreated control) using the following formula (Equation 1):

$$\text{RDW} = [(\text{DW} \times 100)/\text{ADW}] \quad [1]$$

In Equation 1, RDW is the relative dry weight (% of nontreated control), DW is the dry weight of the sample (in grams), and ADW is the average dry weight of the nontreated control (in grams).

Absorption and Translocation of [^{14}C]2,4-D in KCTR, KSS, and MSS *Amaranthus palmeri*

KCTR, KSS, and MSS seedlings were grown (as described earlier), transplanted in the greenhouse, and then moved to growth chambers. Seedlings (10- to 12-cm height) of the three populations were

treated with a stock solution containing a total of 3.3 kBq of ^{14}C-radiolabeled 2,4-D (American Radio Chemicals, St Louis, MO) with a specific activity of 50 mCi mmol^{-1}. For preparing the stock solution, ^{14}C-radiolabeled 2,4-D was mixed with commercial 2,4-D amine (2,4-D Amine 4L, Winfield United Solutions, Arden Hills, MN) to obtain a dose equivalent to the field-recommended dose (560 g ha^{-1}) in a carrier volume of 187 L ha^{-1}. Each plant was treated with 20,000 dpm of the stock solution on the upper surface of the third or fourth fully opened young leaf. The treated plants were returned to their respective growth chambers after 30 min, and plant parts were harvested at four time points: 6, 24, 48, and 72 h after treatment (HAT). At each harvesting time point, the treated plants were trisected into three parts, namely, TL (treated leaf), ATL (area above treated leaf), and BTL (area below treated leaf), to determine the amount of 2,4-D absorbed and translocated within the plant. The treated leaf of each sample was washed with 5 ml wash solution (10% ethanol and 0.05% Tween-20) twice for 1 min to remove excess unabsorbed herbicide. A scintillation cocktail (EcoLite(+)^{TM}, MP Biomedicals, Solon, OH) was added to the leaf rinsate in order to measure the radioactivity in a liquid scintillation counter (LSC; LS 6500 Liquid Scintillation Counter, Beckman Coulter, Brea, CA). Once washed, the trisected plant parts were individually wrapped in wipes (Kimwipes®, Kimberly-Clark Corporation, Roswell, GA), and packed in brown paper envelopes for drying in an oven at 65 C for 72 h. These dried plant parts were combusted using a biological oxidizer (OX-501, RJ Harvey Instrument, Tappan, NY), and [^{14}C]CO_2 generated from the combustion was trapped in a carbon-14 trapping cocktail (RJ Harvey Instrument). There were three replications for each treatment, and the experiment was repeated once. The following equations were used to convert LSC data into percent [^{14}C]2,4-D absorbed and translocated (Equations 2–7):

$$\text{Abs.}(\%) = \left[\left(R_{\text{Applied}} - R_{\text{Rinsate}}\right)/R_{\text{Applied}}\right] \times 100 \quad [2]$$

$$\text{Trans.}(\%) = 100 - \left[R_{\text{TL}}/\left(R_{\text{Applied}} - R_{\text{Rinsate}}\right) \times 100\right] \quad [3]$$

$$\text{TL}(\%) = R_{\text{TL}}/\left(R_{\text{Applied}} - R_{\text{Rinsate}}\right) \times 100 \quad [4]$$

$$\text{ATL}(\%) = R_{\text{ATL}}/\left(R_{\text{Applied}} - R_{\text{Rinsate}}\right) \times 100 \quad [5]$$

$$\text{BTL}(\%) = R_{\text{BTL}}/\left(R_{\text{Applied}} - R_{\text{Rinsate}}\right) \times 100 \quad [6]$$

$$\text{Recovery}(\%) = \left[\left(R_{\text{Rinsate}} + R_{\text{TL}} + R_{\text{ATL}} + R_{\text{BTL}}/R_{\text{Applied}}\right) \times 100 \quad [7]$$

In the above equations, R_{Applied} is the total amount of radioactivity in disintegration per minute (dpm) applied in each plant, Abs. (%) is the percent absorption, R_{Rinsate} is the amount of radioactivity recovered (dpm) in the treated-leaf wash rinsate, Trans (%) is the percent translocation, R_{TL} (%) is the percent radioactivity recovered in the treated leaf, R_{ATL} (%) is the percent radioactivity recovered in the plant tissue above the treated leaf, R_{BTL} (%) is the percent radioactivity recovered in the plant tissue below the treated leaf, and Recovery (%) is the total amount of radioactivity recovered in the experiment.

Metabolism of [^{14}C]2,4-D in KCTR, KSS, MSS Amaranthus palmeri and Wheat

KCTR, KSS, and MSS seedlings of *A. palmeri* were grown as described earlier for absorption and translocation experiments in growth chambers under the same conditions. Additionally, wheat seedlings (winter wheat 'KS Western Star') were grown as a positive control because of their natural ability to metabolize 2,4-D. *Amaranthus palmeri* plants 10- to 12-cm in height and wheat plants at the 3- to 4-leaf stage were treated with [^{14}C]2,4-D stock solution. The stock solution contained 7.2 kBq [^{14}C]2,4-D with a concentration of 30,000 dpm µl^{-1}. For treatment, 10 µl of this [^{14}C]2,4-D stock solution was applied on the adaxial surface of the third or fourth youngest leaf of *A. palmeri* seedlings and the second fully opened leaf of wheat plants. *Amaranthus palmeri* plants were harvested at 4 time points: 6, 24, 48, and 72 HAT. Because wheat is known to rapidly metabolize 2,4-D (deBoer et al. 2006, 2011; Tanetani et al. 2013), seedlings were harvested only at 24 HAT. At each harvest time, treated leaves were washed, as described previously, to remove unabsorbed herbicide, and then each whole seedling was clipped individually aboveground and flash-frozen in liquid nitrogen along with its respective washed treated leaf. The frozen seedlings each were homogenized separately using a mortar and pestle, and [^{14}C]2,4-D and its metabolites were extracted using 15 ml of 90% acetone at 4 C for 16 h. This was followed by centrifugation at 5,000 × *g* for 10 min and concentration of the supernatant at 45 C for 2 h with a rotary evaporator (Centrivap, Labconco, Kansas City, MO) to obtain a final volume of around 600 to 1,000 µl. The supernatant was transferred to a 1.5-ml microcentrifuge tube and centrifuged at 10,000 × *g* for 10 min at room temperature to precipitate the waste, and supernatant was collected. After centrifugation, the radioactivity of each sample was measured using the LSC by adding 2 µl of extract (supernatant) in 15 ml of scintillation fluid (EcoLite(+)^{TM}, MP Biomedicals). The data were used to normalize the sample to a standard concentration of 3,000 dpm 50 µl^{-1} by adding 50% acetonitrile (HPLC grade, Fisher-Scientific, Waltham, MA). About 500 µl of this extract from each sample was run through reverse-phase high-performance liquid chromatography (HPLC; 1260 Infinity II LC System, Agilent, Santa Clara, CA) to quantify the parental compound and metabolites of 2,4-D using a previously standardized method (Figueiredo et al. 2018; Shergill et al. 2018).

Effect of P450 Inhibitors in Metabolizing 2,4-D in KCTR and KSS Amaranthus palmeri

To assess the involvement of P450s in mediating the metabolism of 2,4-D in KCTR, dose–response experiments were conducted in a greenhouse with KCTR and KSS seedlings of *A. palmeri* using P450 inhibitors. These experiments were conducted separately with two P450 inhibitors, that is, malathion and piperonyl butoxide (PBO). For these experiments, treatments were arranged in a factorial structure that included three factors for each inhibitor: *A. palmeri* population (2 levels: KCTR and KSS), inhibitor dose (2 levels: nontreated and treated), and 2,4-D dose (6 levels: nontreated, 140, 280, 560, 1,120, and 2,240 g ha^{-1}). Malathion (Spectracide®, Malathion Concentrate, St Louis, MO), a commonly used P450 inhibitor, was applied 30 min before 2,4-D treatment at 1,500 g ai ha^{-1} and was reapplied as a soil drench (5 mM) at 48 HAT, as described by Ma et al. (2013). Similar to malathion, PBO was applied 30 min before 2,4-D treatment at 2,100 g ai ha^{-1}, as described in previous studies (Shergill et al. 2018; Zhao et al. 2019). Eight replications were maintained for each treatment, and the experiment was repeated once. All inhibitor and 2,4-D applications were done as described earlier for the 2,4-D dose–response experiments.

Table 1. Regression parameters (Equation 1) describing the response of KSS (susceptible), MSS (susceptible), and KCTR (resistant) *Amaranthus palmeri* to 2,4-D under growth-chamber conditions.[a]

Population	*b* (SE)	*d* (SE)	*e* (SE)[b]	RI[n]
			—g ae ha^{-1}—	
KSS	0.67 (0.2)	99.94 (7.4)	126.71 (62.4)	
MSS	0.90 (0.2)	99.82 (7.4)	227.49 (72.8)	
KCTR	0.91 (0.2)	104.75 (6.5)	1441.08 (342.5)	11.37 (compared to KSS); 6.33 (compared to MSS)

[a]Data combined over two runs. SE is the model estimated standard of error. *b* is the relative slope; *d* is the upper limit of the regression model fit; *e* is the estimated GR_{50} or herbicide dose required for 50% dry weight reduction.
[b]RI (resistance index) is the ratio of GR_{50} values of resistant and susceptible populations.

Data Analysis

Relative dry weight data from two runs of each of the 2,4-D dose–response and P450-inhibitor dose–response experiments were tested for homogeneity of variance via Levene's test ($\alpha = 0.05$). Because the test was nonsignificant, the data were pooled. Pooled data were analyzed using a three-parameter log-logistic regression in R utilizing the DRC package (Knezevic et al. 2007; Ritz et al. 2015). The following three-parameter regression model (Equation 8) was fit:

$$Y = d + \exp\left[b(\log x - \log e)\right] \quad [8]$$

In the equation, Y is the response variable, that is, relative dry weight; x is the applied 2,4-D dose; d is the upper limit; b is the relative slope around e; and e is GR_{50}, that is, the amount of 2,4-D required to reduce aboveground dry weight by 50%. Using the *compParm* function in the DRC package, estimated GR_{50} values for *A. palmeri* populations were further tested for difference via a *t*-test (Shyam et al. 2019). The built-in *plot* function in the DRC package was used to obtain the dose–response curves.

Percent [^{14}C]2,4-D absorbed and translocated in the treated plants was determined and the data were fit with asymptotic regression, a rectangular hyperbola (RHB) and linear model, as suggested by Kniss et al. (2011). The analysis was done using the DRC and QPCR packages in R (v. 4.0.3; R Core Team 2020) with the R Studio 9.4 interface (R Studio, PBC, Boston, MA) (Ritz et al. 2015; Spiess 2018). Following the model fitting of the data, the bias-corrected Akaike information criteria (AICc) of each model were compared, and based on the lowest AICc value, the RHB model was selected for analyzing both the absorption and translocation data. The following RHB models were fit (Kniss et al. 2011) (Equations 9 and 10):

$$\text{Abs.} = (A_{max} \times t)/[(10/90) \times A_{90} + t] \quad [9]$$

$$\text{Trans.} = (T_{max} \times t)/[(10/90) \times T_{90} + t] \quad [10]$$

In Equations 8 and 9, Abs. is percent herbicide absorbed, expressed as percentage herbicide applied to the plant (Equation 1); and Trans. is the percent herbicide translocated expressed in terms of percentage herbicide absorbed in the plant (Equation 2). A_{max} and T_{max} are the maximum herbicide absorption and translocation, respectively, in time t, whereas A_{90} and T_{90} are the times required for 90% of the absorption and translocation to occur. Percent [^{14}C]2,4-D translocated in TL, ATL, and BTL (Equations 5–7) were plotted as line graphs using GGPLOT2 (Wickham 2016).

In the metabolism experiments, HPLC chromatograms showing separated parent [^{14}C]2,4-D and polar metabolites of the different *A. palmeri* populations and wheat at different time points were used for visual assessment of 2,4-D metabolism. Percent parent [^{14}C]2,4-D present in each sample was determined based on the area of the chromatograms. Levene's test was conducted using the CAR package in R Studio to compare the two metabolic runs, and because the result was nonsignificant, data were pooled (Fox and Weisberg 2019). Percent parent [^{14}C]2,4-D data were analyzed using two-way ANOVA in R with population (3 levels: KSS, MSS, and KCTR) and time points (4 levels: 6, 24, 48, and 72 HAT) being the two factors. Because the interaction between the two factors was significant, the data were plotted based on each time point for better visualization.

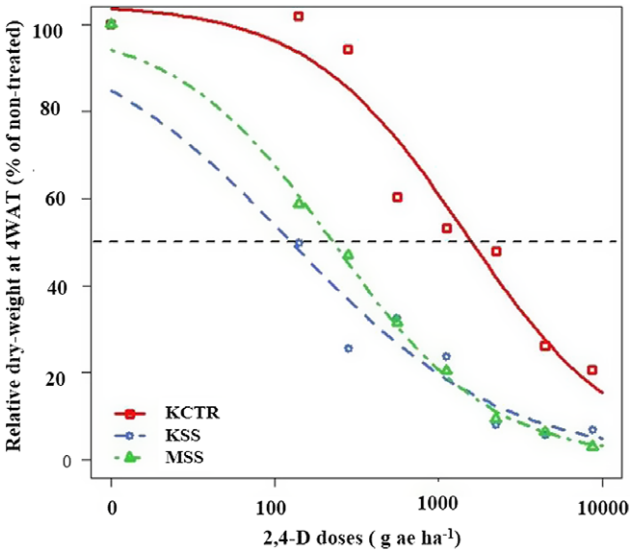

Figure 1. Dose–response curves describing the response of KSS (susceptible), MSS (susceptible), and KCTR (resistant) *Amaranthus palmeri* to 2,4-D. Relative dry weight (% of nontreated) of KSS, MSS, and KCTR *A. palmeri* was analyzed using the three-parameter log-logistic regression model (Equation 1) at 4 wk after treatment (WAT). Dashed line at the center of the plot represents 50% of the relative dry weight.

Results and Discussion

2,4-D Dose–Response Assay

Dose–response analysis revealed that the amount of 2,4-D required to reduce aboveground dry weight by 50% (GR_{50}) at 4 WAT was ~1,441g ha^{-1} for KCTR compared with 127 and 227 g ha^{-1} for KSS and MSS, respectively (Figure 1; Table 1). Even though under controlled environment growth-chamber conditions, susceptible plants survived the field recommended

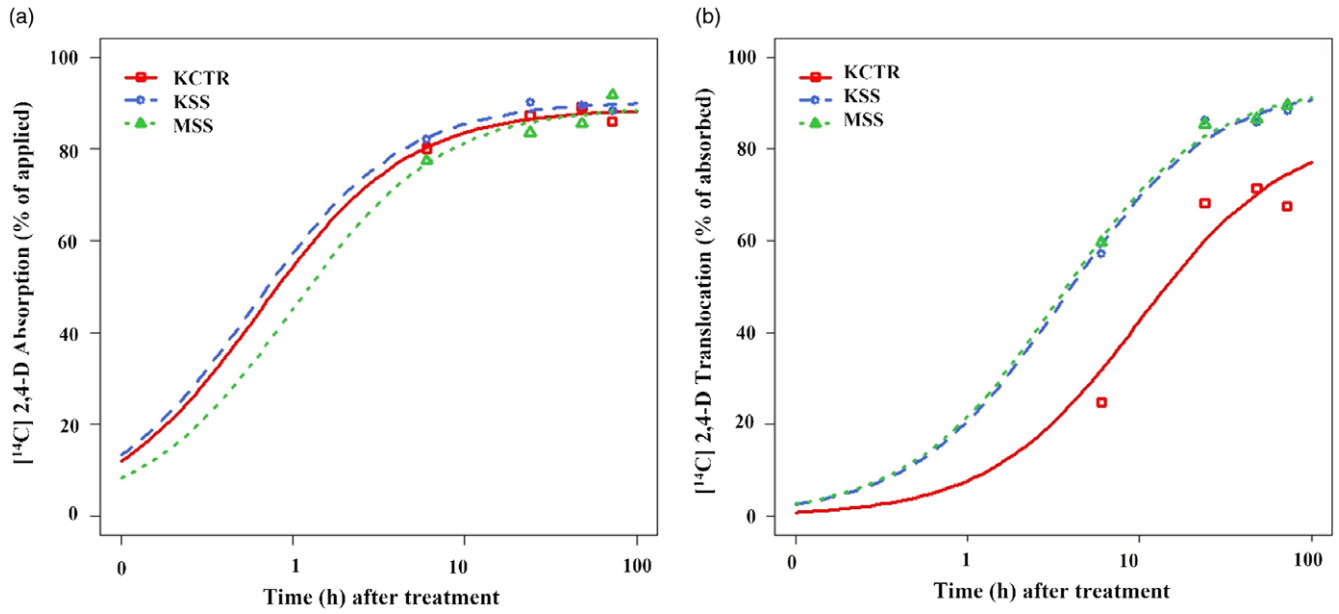

Figure 2. [^{14}C]2,4-D (A) absorption and (B) translocation in KSS (susceptible), MSS (susceptible), and KCTR (resistant) plants grown under growth-chamber conditions, as determined using Equations 9 and 10.

rate of 2,4-D (560 g ha^{-1}) but showed a high level of epinasty and stunting up to 4 WAT. Absolute discrimination in terms of live and dead plants between KCTR and susceptible populations was observed at 1,120 g ha^{-1} in the growth-chamber experiments. Based on GR_{50} values, KCTR was ~11 and ~6 times more resistant compared with KSS and MSS, respectively (Figure 1; Table 1). This level of resistance is moderate and provides insight that the resistance is likely mediated by NTSR mechanisms, because NTSR generally bestows relatively low to moderate levels of resistance to synthetic auxin herbicides. A *t*-test analysis using the *comParm* function in the DRC package in R showed that there was a significant difference in the estimated GR_{50} of KCTR compared with KSS and MSS. So far, the evolution of 2,4-D resistance has been reported in three *Amaranthus* spp.: *A. palmeri*, *A. tuberculatus*, and smooth pigweed (*Amaranthus hybridus* L.) (Heap 2022). Kumar et al. (2019) reported a 3- to 4-fold resistance to 2,4-D in *A. palmeri* compared with susceptible populations.

Absorption and Translocation of [^{14}C]2,4-D in KCTR, KSS, and MSS Amaranthus palmeri

Percent [^{14}C]2,4-D absorption in the experiment ranged from 75% to 90%, and there was no difference between populations. An RHB was utilized to analyze percent [^{14}C]2,4-D absorption and translocation at 6, 24, 48, and 72 HAT in the three *A. palmeri* populations, that is, KCTR, KSS, and MSS. Overall, in all the populations, a maximum of ≥89% [^{14}C]2,4-D was found to be absorbed (A_{max}; Figure 2A; Table 2). There was no statistical difference in the model that estimated A_{max} (maximum percent absorption) of KCTR, KSS, and MSS *A. palmeri* (Tables 2 and 3). The time required to achieve A_{max} (i.e., A_{90}) in the three populations varied from 5 to 9 h (Figure 2A; Table 2). Even though MSS took a longer time to achieve maximum absorption of [^{14}C]2,4-D compared with KCTR and KSS, the *t*-test analysis showed no statistical difference in estimated A_{90} of KCTR, KSS, and MSS (Table 3). The results confirm that differential 2,4-D absorption is not contributing to 2,4-D resistance in KCTR *A. palmeri*. Analysis of percent translocation suggests that KSS, MSS, and KCTR translocated a maximum (T_{max}) of 93.9%, 94.2%, and 84.6% [^{14}C]2,4-D, respectively (Table 2). Hence, there was ~10% higher translocation of [^{14}C]2,4-D in KSS and MSS compared with KCTR, which was statistically significant (Tables 2 and 3). Overall, >84% of absorbed [^{14}C]2,4-D was translocated in all three populations (Figure 2B; Table 2). Time required to translocate the maximum amount of [^{14}C]2,4-D (T_{90}) in KCTR was much higher (~89 h) than in KSS and MSS (~30 h), indicating slower translocation of 2,4-D compared with susceptible plants (Figure 2B; Table 2). For T_{90}, the difference between KCTR and KSS was significant at $\alpha = 0.1$, while the difference between KCTR and MSS was significant at $\alpha = 0.05$ level (Table 3). For all populations, a higher quantity of [^{14}C]2,4-D was translocated toward tissue below the treated leaf compared with tissue above the treated leaf (not shown). No other population-specific pattern was observed in [^{14}C]2,4-D movement in tissue above or below the treated leaf (not shown). However, ~10% less translocation and ~3 times slower [^{14}C]2,4-D translocation were found in KCTR compared with KSS and MSS plants (Figure 2B; Table 2). Considering that the three populations absorbed ≥89% of [^{14}C]2,4-D applied and ≥84% of that was translocated, this difference in 2,4-D translocation is unlikely to significantly contribute to the resistance. Further, because our data also suggest that the KCTR plants can metabolize 2,4-D faster than the susceptible plants, the 2,4-D translocation in KCTR plants may also include metabolites that may contribute to slower translocation of 2.4-D.

Previously, no difference in [^{14}C]2,4-D absorption or translocation was reported in 2,4-D–resistant *A. tuberculatus* populations from Nebraska and Missouri (Figueiredo et al. 2018; Shergill et al. 2018). Nonetheless, differential 2,4-D uptake and/or translocation was reported to impart 2,4-D resistance in several weeds such as *P. rhoeas*, *A. hybridus*, parthenium (*Parthenium hysterophorus* L.), shortpod mustard [*Hirschfeldia incana* (L.) Lagr.-Foss.], and *R. raphanistrum* (Goggin et al. 2016; Rey-Caballero et al. 2016). Influx (AUX1/LAX1) and efflux (PIN/ABCB) auxin

Table 2. Parameters describing percent absorption of [^{14}C]2,4-D in KSS, MSS, and KCTR *Amaranthus palmeri* plants calculated from Equations 9 and 10.[a]

Population	Percent [^{14}C]2,4-D absorption		Percent [^{14}C]2,4-D translocation	
	A_{max} (SE)	A_{90} (SE)	T_{max} (SE)	T_{90} (SE)
	—%—	—h—	—%—	—h—
KSS	90.41 (1.8)	5.21 (2.3)	93.91 (4.5)	31.87 (11.1)
MSS	89.27 (1.8)	8.78 (2.7)	94.21 (4.2)	30.11 (8.7)
KCTR	88.77 (1.8)	5.70 (2.4)	84.63 (6.4)	89.17 (26.4)

[a]Data combined over two runs. SE is the model estimated standard of error. A_{max} and T_{max} are the maximum limit of [^{14}C]2,4-D absorption (%) and translocation (%) over time *t* in each population. A_{90} and T_{90} refer to the amount of time (hours) required for that maximum absorption and translocation to occur.

Table 3. Comparison of parameters estimating [^{14}C]2,4-D absorption and translocation in KSS (susceptible), MSS (susceptible), and KCTR (resistant) *Amaranthus palmeri* plants (using Equations 9 and 10) when grown under growth-chamber conditions.

Parameters[a]	Population comparisons[b]		
	KSS vs. MSS	KCTR vs. MSS	KSS vs. KCTR
		P-value	
A_{max}	0.660^{NS}	0.848^{NS}	0.553^{NS}
A_{90}	0.319^{NS}	0.397^{NS}	0.884^{NS}
T_{max}	0.305^{NS}	0.215^{NS}	0.247^{NS}
T_{90}	0.901^{NS}	0.039**	0.051*

[a]A_{max} and T_{max} are the maximum limit of [^{14}C]2,4-D absorption (%) and translocation (%) over time *t* in each population. A_{90} and T_{90} refer to the amount of time (hours) required for that maximum absorption and translocation to occur.
[b]A *t*-test was performed to compare parameters of each population. Data combined over two runs. P-values: *P < 0.1; **P < 0.05; ***P < 0.01; NS, nonsignificant. These symbols indicate the level of significance of difference in means.

transporters play a key role in directional transport of auxins (Cho and Cho 2013; Fischer et al. 1998; Petrášek and Friml 2009). Because SAHs mimic several physiological processes of natural plant hormones, alteration of auxin transporters can lead to decreased SAH transport, resulting in reduced translocation of SAHs. However, no field-evolved resistance to SAH caused by alterations in the auxin transporters was reported. Interestingly, application of auxin transport inhibitors, such as 2,3,5-triiodobenzoic acid and 1-*N*-naphthylphthalamic acid, to 2,4-D–susceptible *R. raphanistrum* from Australia resulted in a reduced 2,4-D translocation, mirroring the 2,4-D–resistant *R. raphanistrum* (Goggin et al. 2016).

Metabolism of [^{14}C]2,4-D in KCTR, KSS, MSS *Amaranthus palmeri* and Wheat

To investigate whether enhanced metabolism of 2,4-D confers resistance in KCTR, the rate of [^{14}C]2,4-D metabolism over time was assessed in KSS, MSS, and KCTR seedlings. The extracts of [^{14}C]2,4-D–treated seedlings of KSS, MSS, and KCTR *A. palmeri* were resolved into parent [^{14}C]2,4-D and its polar metabolites using reverse-phase HPLC. Wheat seedlings were used as a positive control, because of their natural ability to metabolize 2,4-D. In the chromatograms, a single peak with a retention time (RT) of the parent [^{14}C]2,4-D was found at ~10.5 min (Figure 3A). Apart from parent [^{14}C]2,4-D, three major polar metabolites were identified from these samples: metabolite 1 (M1, RT= ~7.9 min), metabolite 2 (M2, RT= ~6.3 min), and metabolite 3 (M3, RT= ~5.5 min), respectively (Figure 3B and C). As expected, wheat seedlings rapidly metabolized 2,4-D, with ~70% of [^{14}C]2,4-D degraded by 24 HAT (Figure 3D). Based on RT, chromatograms of wheat samples at 24 HAT showed the presence of the [^{14}C]2,4-D metabolites with polarity and retention times similar to those seen in KCTR (Figure 3C). There was no difference in the amount of [^{14}C]2,4-D metabolized in KSS or MSS and KCTR at 6 HAT (Figure 4A). However, at 24, 48, and 72 HAT, there was ~20% to 30% significantly higher parent [^{14}C]2,4-D in KSS and MSS compared with KCTR (Figure 4B–D).

Enhanced metabolism has been shown to impart 2,4-D resistance in *A. tuberculatus* populations in the U.S. Midwest (Figueiredo et al. 2018; Shergill et al. 2018). Metabolism of 2,4-D has also been documented in *P. rhoeas* populations from Spain and *A. hybridus* populations from Argentina (Dellaferrera et al. 2018; Rey-Caballero et al. 2016; Torra et al. 2017). Although limited information is available regarding how metabolites of 2,4-D are translocated in plants, our data suggest reduced translocation (~10%) of 2,4-D in KCTR plants compared with KSS or MSS (Table 3). Because KCTR plants metabolize 2,4-D at 24, 48, and 72 HAT (Figure 4), it is possible that the translocated compounds include hydroxylated (Phase I) metabolites of 2,4-D, which are more likely to be translocated rather than the parent compound. This possibility needs to be investigated and confirmed. Metabolites with similar polarity and retention time were found in wheat and KCTR *A. palmeri* (Figure 3). This suggests that KCTR *A. palmeri* might be able to metabolize 2,4-D through the same biochemical process as wheat. The 2,4-D metabolites in wheat (and other monocots) are usually considered to be stable-sugar conjugates and nontoxic. However, amino acid conjugates are less stable than sugar conjugates and can be reversed to form an active form of the parent herbicide compound (2,4-D) that is toxic (Sterling and Hall 1997). Jablonkai (2015) reported that in wheat, 2,4-D is primarily metabolized through aromatic-ring hydroxylation of the 2,4-dichloro-phenyl moiety, and an acidic aryl glycoside is subsequently formed by conjugation. Future investigations will be directed toward identification of 2,4-D metabolites in KCTR and wheat through the application of liquid chromatography–mass spectrometry techniques. This will shed light on the potential biochemical pathways through which 2,4-D is metabolized in KCTR *A. palmeri*.

Effect of P450 Inhibitors on KSS and KCTR *Amaranthus palmeri*

For testing the involvement of P450s in mediating 2,4-D metabolism in KCTR *A. palmeri*, we conducted dose–response experiments using two known P450 inhibitors (malathion and PBO). In the absence of malathion, the GR_{50} values for KSS and KCTR were 187 and 1,572 g ha^{-1}, respectively (Figure 5; Table 4). However, when pretreated with malathion followed by 2,4-D, the GR_{50} values for KSS and KCTR were 197 and 627 g ha^{-1}, respectively (Figure 5; Table 4). Thus, the addition of malathion resulted in a 60% reduction of GR_{50} in KCTR but failed to impact the response of KSS *A. palmeri* to 2,4-D application (Figure 5; Table 4). A *t*-test analysis using the *comParm* function in R showed that there was a significant difference in estimated GR_{50} of KCTR with the application of malathion before 2,4-D treatment compared with 2,4-D applied alone, while such a difference was not

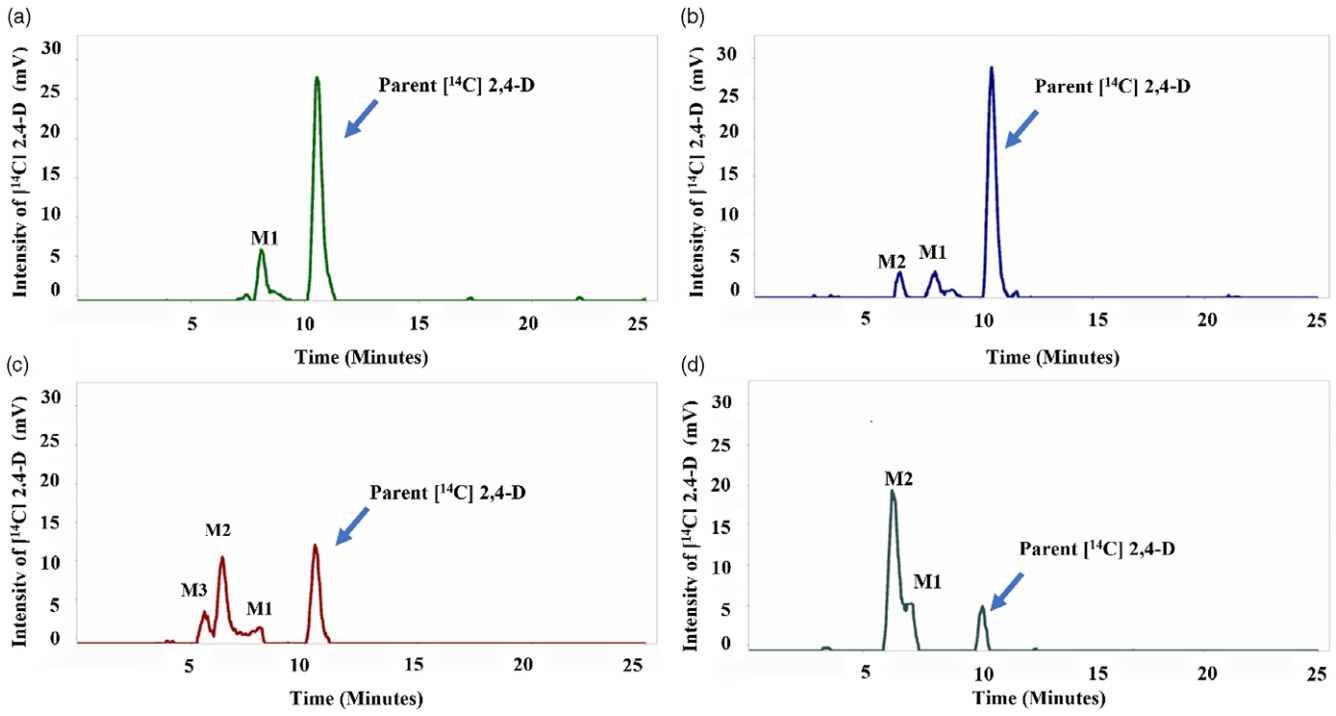

Figure 3. HPLC chromatograms depicting resolved [^{14}C]2,4-D parent compound and its metabolites at 24 h after treatment (HAT) in (A) KSS (susceptible), (B) MSS (susceptible), (C) KCTR (resistant) *Amaranthus palmeri* and (D) wheat (naturally tolerant) seedlings, grown under growth-chamber conditions. M1, M2, and M3 represent metabolites 1, 2, and 3.

present in the case of KSS (Figure 5; Table 4). Unlike the treatment with malathion, the application of PBO did not have any effect on the level of 2,4-D resistance in KCTR (Figure 6; Table 5). The GR_{50} values for KSS and KCTR after 2,4-D application were estimated as 257 and 2,244 g ha^{-1}, respectively (Figure 6; Table 5). Upon pretreatment with PBO, followed by 2,4-D application, the GR_{50} values of KSS and KCTR were estimated as 202 and 2,600 g ha^{-1}, respectively (Figure 6; Table 5). Similar to malathion, the addition of PBO did not significantly impact the response of KSS to the 2,4-D treatment (Figure 6; Table 5). Overall, with the addition of PBO, there was only a 16% difference in GR_{50} of KCTR; however, the value was not statistically significantly different from that for plants treated with only 2,4-D (Figure 6; Table 5). Previously, we have confirmed that KCTR plants treated with P450 inhibitors alone do not show any reduction in the biomass accumulation (Shyam et al. 2021).

These results suggest that there is potential involvement of P450s in mediating metabolic resistance to 2,4-D in KCTR *A. palmeri*. Malathion is commonly used to test for the involvement of P450s in metabolic resistance to herbicides in weeds. P450 enzymes metabolize xenobiotics, such as herbicides, via oxygenation and NADPH-dependent monooxygenation reactions (Yuan et al. 2007). When organophosphate insecticides, such as malathion, react with P450s, they generate atomic sulfur and bind to P450 apoprotein, thereby inactivating its activity (Werk-Reichhart et al. 2000). Therefore, the application of malathion inhibits P450-dependent metabolism and increases the metabolic half-life of herbicides (Kreuz and Fonné-Pfister 1992). Previously, the application of malathion reversed metabolic resistance to 2,4-D in 2,4-D–resistant *A. tuberculatus* (Figueiredo et al. 2018; Shergill et al. 2018). We have already shown that malathion treatment reduces the level of mesotrione, 2,4-D, and lactofen resistance in KCTR *A. palmeri* (Shyam et al. 2021). However, such an impact on 2,4-D resistance in KCTR was not seen with PBO application; the GR_{50} values of KCTR and KSS were not significantly altered with this treatment (Figure 6; Table 5). This indicates that P450s that are potentially not inhibited by PBO are involved in mediating 2,4-D resistance in KCTR. The application of PBO decreased the level of metabolic resistance to herbicides in other weeds, such as metribuzin resistance in rigid ryegrass (*Lolium rigidum* Gaudin) (Ma et al. 2020). Nonetheless, the selectivity of P450 inhibitors was recorded in several metabolically resistant weed populations. For instance, in chlorsulfuron-resistant *L. rigidum*, malathion treatment increased toxicity of chlorsulfuron, but application of tetcyclasis (another P450 inhibitor) did not (Christopher et al. 1992). Dimaano and Iwakami (2021) have suggested that P450-mediated herbicide metabolism can be attributed to a single nucleotide polymorphism, increased copy number, or even changes in gene regulation. However, despite the known involvement of P450s, it is a challenge to identify and isolate specific P450s that are involved in herbicide metabolism. Recently, a P450, *CYP81A10v7* was confirmed to mediate metabolic resistance to ACCase, ALS, PSII, HPPD, and very-long-chain fatty-acid inhibitors in *L. rigidum* (Han et al. 2021). Another P450 cluster (*P450 81E8*) was found to be overexpressed in two different 2,4-D–resistant *A. tuberculatus*

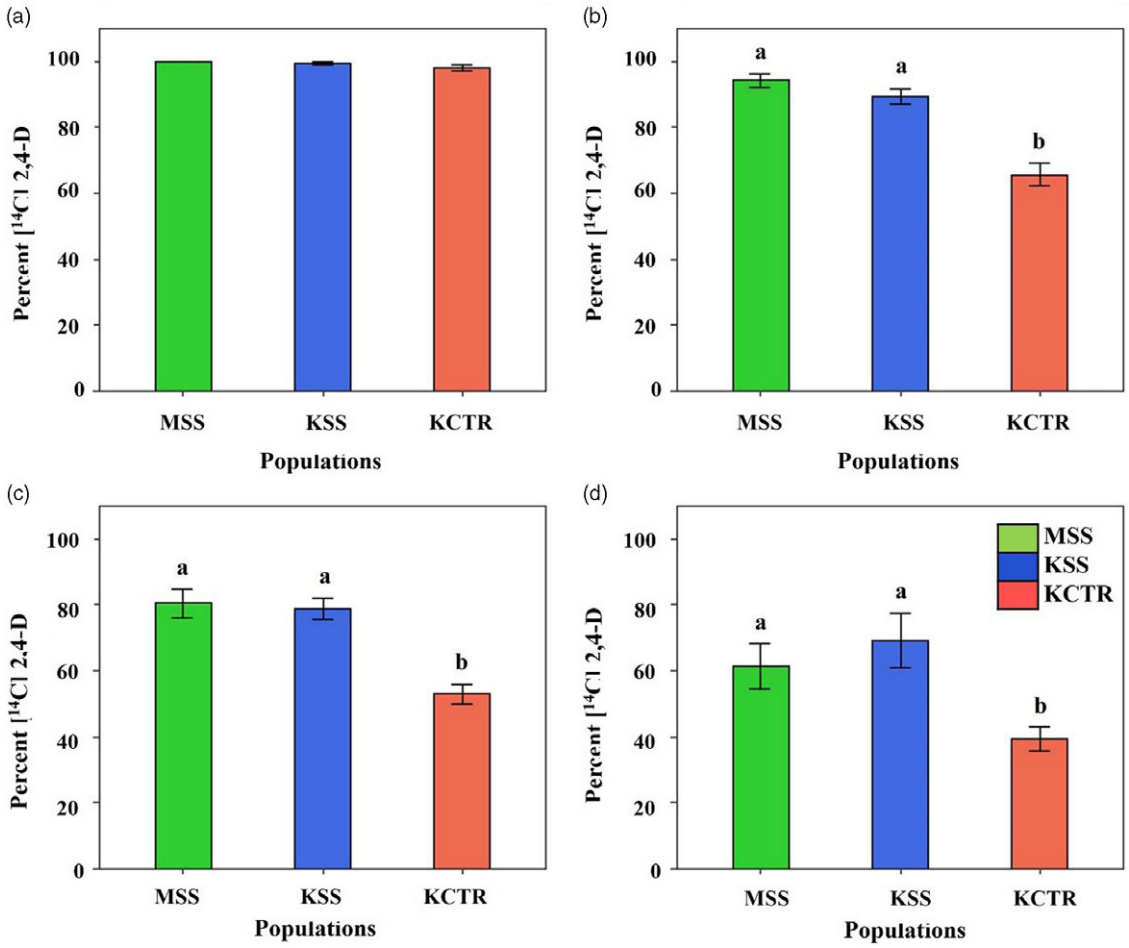

Figure 4. Percent of [^{14}C]2,4-D in KSS (susceptible), MSS (susceptible), and KCTR (resistant) *Amaranthus palmeri* seedlings at (A) 6, (B) 24, (C) 48, and (D) 72 h after treatment (HAT) grown under growth-chamber conditions. Error bars represent standard error of mean and letters represent significant differences identified by separation of means using Tukey's test (5%).

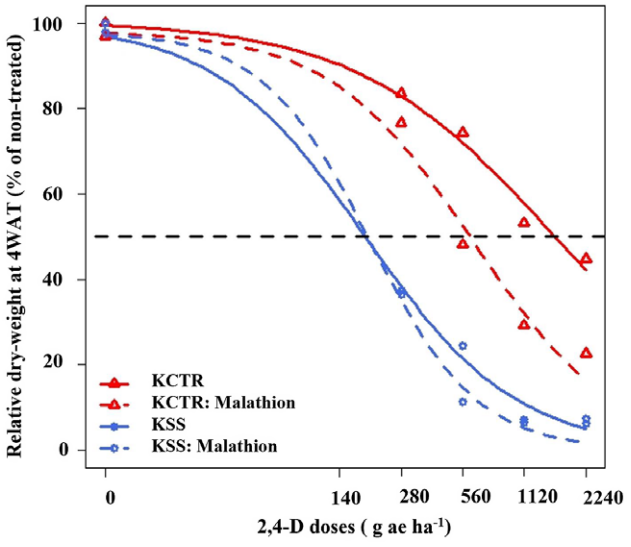

Figure 5. Dose–response curves describing the response of KSS (susceptible) and KCTR (resistant) *Amaranthus palmeri* to 2,4-D with or without pretreatment with malathion (cytochrome P450 inhibitor). Relative dry weight (% of nontreated) of KSS and KCTR *A. palmeri* was analyzed using the three-parameter log-logistic regression model (Equation 1) at 4 wk after treatment (WAT). Dashed line at the center of the plot represents 50% of the relative dry weight.

populations from Nebraska and Illinois (Giacomini et al. 2020). Therefore, it will be important to test the expression and activity of these P450 genes and enzymes in KCTR before and after 2,4-D application.

Summary and Implications

Overall, the results provide evidence that metabolism of 2,4-D bestows resistance in KCTR *A. palmeri*. Pretreatment with a P450 inhibitor, malathion, before 2,4-D application, but not pretreatment with PBO, resulted in reversal of 2,4-D resistance and increased efficacy of 2,4-D in KCTR *A. palmeri*. The possible presence of TSR mechanisms leading to 2,4-D resistance in KCTR *A. palmeri* is yet to be studied. Metabolic resistance to herbicides is currently one of the most complex challenges affecting the sustainability of crop production. The ability of metabolic resistance to confer cross-resistance to herbicides with multiple SOAs, as seen in the KCTR *A. palmeri*, is especially challenging. This is the first instance of metabolic resistance to 2,4-D in *A. palmeri*. So far, evolution of 2,4-D resistance in Kansas populations has not been reported to be widespread. Therefore, it will be important to adhere to best management practices, including integrated weed management and rotation of herbicides targeting different SOAs, to discourage such an occurrence of resistance.

Table 4. Regression parameters (Equation 1) describing the response of KSS (susceptible) and KCTR (resistant) *Amaranthus palmeri* under growth-chamber conditions to 2,4-D application with or without pretreatment with malathion (cytochrome P450 inhibitor).[a]

Population	Treatment	*b* (SE)	*d* (SE)	*e* (SE)	Reduction	P-value[b]
				—g ae ha^{-1}—	—%—	
KSS	2,4-D	1.18 (0.26)	99.96 (4.49)	187.39 (41.77)	−11	0.8705^{NS}
	Malathion followed by 2,4-D	1.65 (0.55)	97.92 (4.82)	197.39 (44.80)		
KCTR	2,4-D	0.89 (0.23)	100.44 (5.99)	1572.47 (370.50)	60	0.0129**
	Malathion followed by 2,4-D	1.25 (0.18)	98.14 (4.36)	626.56 (74.67)		

[a]Data combined over two runs. SE is the model estimated standard error. *b* is the relative slope; *d* is the upper limit of the regression model fit; *e* is the estimated GR_{50} or herbicide dose required for 50% dry weight reduction.
[b]P-values: **P < 0.05; NS, nonsignificant. These symbols indicate the level of significance of mean difference of GR_{50}.

Table 5. Regression parameters (Equation 7) describing the response of KSS (susceptible) and KCTR (resistant) *Amaranthus palmeri* to 2,4-D with or without pretreatment with piperonyl butoxide (PBO; cytochrome P450 inhibitor) under growth-chamber conditions.[a]

Population	Treatment	*b* (SE)	*d* (SE)	*e* (SE)	Reduction	P-value[b]
				—g ae ha^{-1}—	—%—	
KSS	2,4-D	1.68 (0.41)	100.06 (4.81)	257.25 (37.61)	−27	0.3216^{NS}
	PBO followed by 2,4-D	1.49 (0.39)	99.99 (4.99)	202.22 (40.71)		
KCTR	2,4-D	0.68 (0.19)	99.99 (4.64)	2244.12 (630.25)	16	0.7498^{NS}
	PBO followed by 2,4-D	0.67 (0.17)	100.08 (4.61)	2600.49 (921.62)		

[a]Data combined over two runs. SE is the model estimated standard error. *b* is the relative slope; *d* is the upper limit of the regression model fit; *e* is the estimated GR_{50} or herbicide dose required for 50% dry weight reduction.
[b]NS, nonsignificant; indicates the level of significance of mean difference of GR_{50}.

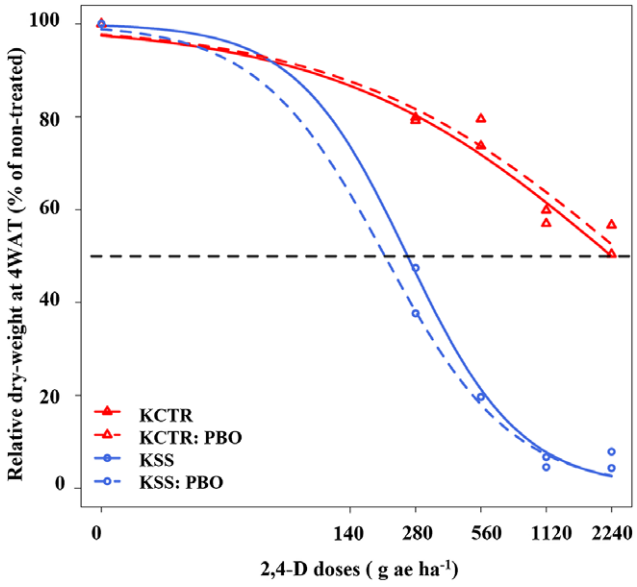

Figure 6. Dose–response curves describing the response of KSS (susceptible) and KCTR (resistant) *Amaranthus palmeri* to 2,4-D with or without pretreatment with piperonyl butoxide (PBO; cytochrome P450 inhibitor). Relative dry weight (% of nontreated) of KSS and KCTR *A. palmeri* was analyzed using the three-parameter log-logistic regression model (Equation 1) at 4 wk after treatment (WAT). Dashed line at the center of the plot represents 50% of the relative dry weight.

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