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Metabolism of 2,4-dichlorophenoxyacetic acid contributes to resistance in a common waterhemp (*Amaranthus tuberculatus*) population

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Running title: Enhanced 2,4-D metabolism in *A. tuberculatus*

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Abstract:

BACKGROUND: Synthetic auxins such as 2,4-D have been widely used for selective control of broadleaf weeds since the mid-1940s. In 2009, an *Amaranthus tuberculatus* (common

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waterhemp) population with 10-fold resistance to 2,4-D was found in Nebraska, USA. The 2,4-D resistance mechanism was examined by conducting [^{14}C] 2,4-D absorption, translocation and metabolism experiments.

RESULTS: No differences were found in 2,4-D absorption or translocation between the resistant and susceptible *A. tuberculatus*. Resistant plants metabolized [^{14}C] 2,4-D more rapidly than did susceptible plants. The half-life of [^{14}C] 2,4-D in susceptible plants was 105 h, compared to 22 h in resistant plants. Pre-treatment with the cytochrome P450 inhibitor malathion inhibited [^{14}C] 2,4-D metabolism in resistant plants and reduced the 2,4-D dose required for 50% growth inhibition (GR_{50}) of resistant plants by 7-fold to 27 g ha^{-1}, similar to the GR_{50} for susceptible plants in the absence of malathion.

CONCLUSIONS: Our results demonstrate that rapid 2,4-D metabolism is a contributing factor to resistance in *A. tuberculatus*, potentially mediated by cytochrome P450. Metabolism-based resistance to 2,4-D could pose a serious challenge for *A. tuberculatus* control due to the potential for cross-resistance to other herbicides.

Key words: 2,4-D resistance, 2,4-D metabolism, *Amaranthus tuberculatus*, 2,4-D uptake and translocation, cytochrome P450.

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1 INTRODUCTION

The synthetic auxin herbicide 2,4-D was introduced for weed control in agriculture in the mid-1940s[1] and has since become one of the most widely used herbicides in the world. This and other auxinic herbicides are popular among growers, in part because of their ability to selectively control broadleaf weeds. In 2005, the United States Environmental Protection Agency estimated annual 2,4-D use in agriculture and non-agriculture settings at 20.9 million kg.[2] Even after the introduction of newer herbicides, such as glyphosate, triazines, and acetolactate synthase (ALS) inhibitors, auxinic herbicide use has remained high, primarily because of their selectivity, efficacy, broad-spectrum of control, and low cost.[1] More recently, the widespread and increasing evolution of resistance in weed species to various other herbicides has resulted in an increase in auxinic herbicide use. The development and commercialization of 2,4-D-resistant cotton (*Gossypium hirsutum*) and soybean (*Glycine max*) crop varieties[3] will likely increase 2,4-D use for in-crop selective weed control.

Synthetic auxin herbicides are known to mimic several physiological and biochemical responses induced by the natural plant hormone, indole acetic acid (IAA).[4] Despite their extensive use in agriculture for several decades, the precise mechanism of synthetic auxin herbicide action is not completely understood. Upon discovery of IAA receptors Transport Inhibitor Response 1 (TIR1) and Auxin F-Box (AFB) proteins,[5,6] the role of these proteins in auxinic herbicide-mediated responses has also been examined.[7,8] One hypothesis is that functional redundancy in auxin receptors (i.e., TIR1 and AFBs 1-5) might contribute to multiple sites of action for auxinic herbicides. The precise role of these proteins in auxinic herbicide-mediated responses is still elusive. Previous research also suggests that auxinic herbicides

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activate metabolic processes that initiate ethylene accumulation, resulting in epinasty.[4] Other factors potentially leading to plant death include abscisic acid (ABA) accumulation resulting in 1) photosynthesis inhibition, 2) H_2O_2 production, and 3) increase in reactive oxygen species (ROS).[4,9]

The selectivity of auxinic herbicides in controlling broadleaf species is primarily due to auxinic herbicide metabolism by tolerant species.[10] Metabolism also plays a key role in conferring resistance to these herbicides in dicot species as well.[11] In most cases, auxinic herbicides undergo oxidation, hydrolysis, or conjugation resulting in reduced biological activity.[11-13] In tolerant monocots, metabolic reactions typically occur through ring hydroxylation followed by irreversible glucose conjugation.[14] In sensitive dicots, auxinic herbicides may be conjugated to amino acids, which are reversible to active forms and may still have partial herbicidal activity themselves.[15]

Amaranthus tuberculatus (Moq.) Sauer var. *rudis* (Sauer) Costea and Tardif (common waterhemp) is a major troublesome weed of cropping systems in North America.[16] Especially in agricultural fields of the Midwestern United States, this weed poses a serious problem causing significant yield losses in maize (*Zea mays*) and soybean.[17-20] *A. tuberculatus* is dioecious and a prolific seed producer, which enables rapid spread.[20] High genetic variability coupled with intense herbicide selection pressure has resulted in evolution of resistance to several commonly used herbicides in *A. tuberculatus*.[21-23] US Midwestern populations of *A. tuberculatus* have various combinations of herbicide resistance spanning six modes of action including photosystem II (PSII)-inhibitors, ALS-inhibitors, protoporphyrinogen oxidase (PPO) inhibitors, 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors, 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) inhibitors, and 2,4-D.[24]

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Herbicide resistance has become a major global issue and numerous agriculturally important weeds have confirmed resistance to multiple herbicide modes of action.[24] Even after several decades of continuous auxinic herbicide use, the rate of resistance evolution to auxinic herbicides is comparatively low.[25] There are currently 34 weed species known to have evolved resistance to auxinic herbicides,[24] including *A. tuberculatus*. In 2009, the first failure to control *A. tuberculatus* with 2,4-D was reported in Nebraska, USA. This population was confirmed to have evolved resistance to 2,4-D with a resistance ratio of 10 relative to a susceptible population.[26] The resistance mechanism in this *A. tuberculatus* population has not been determined. The objective of this research was to examine [^{14}C] 2,4-D uptake, translocation, and metabolism in an effort to identify the resistance mechanism.

2 MATERIALS AND METHODS

The 2,4-D-resistant *A. tuberculatus* from southeast Nebraska was used in this research.[26] This population was found in a seed production field of little bluestem (*Schizachyrium scoparia* Michx. Nash) that had been in no-till management with annual application of 2,4-D for over 10 years. The 2,4-D resistant *A. tuberculatus* seed was collected from the field followed by one generation of 2,4-D selection in the greenhouse to produce the seed used in these studies. An *A. tuberculatus* population from Nebraska known to be susceptible to 2,4-D was also used for comparison.

2.1 [^{14}C] 2,4-D Absorption and Translocation

Resistant and susceptible *A. tuberculatus* seeds were planted on potting soil**,** kept in a 4°C room for one week and then transferred to a greenhouse with controlled conditions at 25 ^{o}C and 75% RH until reaching 8 cm or 4 true leaves. Plants were then transplanted to fine washed silica,

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irrigated with fertilizer (0.05% Miracle-Gro solution, Scotts Miracle-Gro Company, Marysville, OH), and transferred to a growth chamber under the same conditions as the greenhouse except for the lighting, which was supplied with fluorescent and incandescent light.

Plants were treated at the stage of 4-6 true leaves (1 wk after transplanting). The fourth true leaf was marked and covered with aluminum foil. Plants were then sprayed in a single nozzle overhead track sprayer (DeVries Generation III Research Sprayer, Hollandale, MN, USA) with 500 g ha^{-1} 2,4-D (2,4-D amine, 455 g L^{-1}, DuPont) in a water volume of 224 L ha^{-1} containing 1% COC. The aluminum foil was then removed and a solution of [^{14}C]-2,4-D mixed with formulated 2,4-D and COC was applied using 10 droplets of 1 µl each, so that the treated leaf received the same amount of herbicide as the rest of the plant (5 µg cm^{-2} and 3 µl cm^{-2}). Total radioactivity applied per plant was 3.33 KBq (200,000 dpm). Three replications per time point were used, and the experiment was repeated.

Evaluation time points were at 12, 24, 48, 96, and 192 HAT. The treated leaves were cut and washed with 5 ml of 10% methanol and 1% NIS washing solution. The leaf rinse solution was mixed with 10 ml of scintillation cocktail (Ecoscint™ XR) and measured for radioactivity using LSS (Packard Tri-carb 2300TR). Roots were washed with 10 ml water, and 3 ml of the wash solution was measured with LSS. Plants including treated leaves were pressed in newspaper and dried in a 60°C oven for 72 h before exposure to Phosphor Screen film for 3 d followed by imaging with a Typhoon Trio Imager (GE Healthcare). The dried tissue was separated into treated leaf, untreated leaves, stem, and roots, and then oxidized in a Biological oxidizer (OX500) followed by radioactivity measurement with LSS. The proportion of absorbed herbicide was calculated using the following equation:

$$\%H_{abs} = [(^{14}C\ ot) / (^{14}C\ ot + ^{14}C\ wl)] \times 100$$

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where "$\%H_{abs}$" is the proportion of absorbed herbicide, "^{14}C ot" is the amount of ^{14}C measured in oxidized tissue, and "^{14}C wl" is the amount of ^{14}C detected in the treated leaf. For herbicide translocation studies the following equation was used:

$\%H_{tr} = 100 - [(^{14}\text{C al}) / (^{14}\text{C al} + {}^{14}\text{C ot}) \times 100]$

where "$\%H_{tr}$" is the proportion of translocated herbicide, "^{14}C al" is the amount of ^{14}C measured in the treated leaf, and "^{14}C ot" is the amount of ^{14}C detected in other untreated tissues of the plant.

2.2 [^{14}C] Metabolism

Plants were treated with the same procedures and conditions as the absorption and translocation studies. They were harvested at 12, 24, 48, 96, 192, and 264 HAT and at each time point, treated leaf, roots, and sand were washed and the plant tissue was rapidly frozen in liquid nitrogen and stored at -80 ºC. Metabolite extraction was performed by grinding the entire plant with a mortar and pestle, then digesting tissue with a 10 ml solution of 1% acetic acid in 50 ml plastic tubes on a table shaker for 10 min. Extracts were put in 50 ml centrifuge filters with 25 ml microfiltration membranes (pore size of 0.45 μm), then the tissue digestion step was repeated two more times. Filters and tissue were dried and kept for oxidation to quantify the non-extracted metabolites. Final extracted volume of 30 ml was applied to a solid phase extraction C18 cartridge, and 5 ml of digestion solution that passed through the cartridge was quantified by LSS. About 95% of radioactivity interacted with the silica matrix and was recovered with 4 ml of acetonitrile and dried in an evaporation system under vacuum at 40 ºC. Entire extracts were suspended in 225 μl of HPLC A solvent and filtered in 1.5 ml centrifuge tubes with 0.4 μm

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microfiltration membranes at 12,000×g. Filtered solution (200 µl) was used for HPLC (Hitachi Instruments, Inc., San Jose, CA) using a C18 4.6 mm by 150 mm column (C18 Column, Zorbax Eclipse XDB-C18, Agilent Technologies, Santa Clara, CA, USA), attached to a radio-detector (FlowStar LB 513, Berthold Technologies GmbH & Co.) with a flow cell YG-150-U5D solid cell YG-Scintillator (150 µl). Mobile Phase A contained 89.9% water, 10% acetonitrile, and 0.1% formic acid and phase B contained 99.9% acetonitrile and 0.1% formic acid. A calibration curve for radioactivity detection was constructed using a series of different counts of [^{14}C]-2,4-D (8.3 Bq, 16.7 Bq, 83.3 Bq, 166.7 Bq, 1666.7 Bq, and 3333.3 Bq). The proportion of 2,4-D metabolism was calculated using the equation:

$\%2,4\text{-}D_{Parent}$ = [(HPLC detected 2,4-D) / (HPLC detected 2,4-D + HPLC detected metabolites + counts in oxidized filters + counts in digestion solution after C18 cartridge separation + counts in washed sand)] ×100

where "$\%2,4\text{-}D_{Parent}$" is the proportion of non-metabolized herbicide. The experiment had 3 replications and it was repeated.

2.3 Malathion Effects on 2,4-D Resistance and Metabolism

Resistant and susceptible *A. tuberculatus* plants were grown in a greenhouse under controlled conditions as described above, except that plants were grown in potting soil. Half of the resistant and susceptible plants were treated with malathion (Spectracide, United Industries Corporation, St. Louis, MO) at 2,000 g ha^{-1}, 24 h before 2,4-D treatment. Plants were treated with 2,4-D (2,4-D amine, 455 g L^{-1}, DuPont) at the developmental stage of 4-5 true leaves and treatments were 0, 15, 30, 63, 125, 250, 500, 1,000, 2,000, and 6,000 g ha^{-1}. Plants were harvested 28 d after treatment and dried in a 60 ^{o}C oven before weighing.

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Another study to analyze malathion effects on 2,4-D metabolism was conducted as described above. Half of the plants transplanted to fine silica were sprayed with malathion at 2,000 g ha^{-1}, and at 24 HAT all resistant and susceptible plants were treated with [^{14}C] 2,4-D as described above. After 264 h, [^{14}C] 2,4-D treated leaves and roots were washed and the tissue was frozen with liquid nitrogen for metabolite extraction as described above. The amount of 2,4-D recovered was calculated using the equation "%$2,4\text{-}D_{Parent}$" described above. Each treatment had 3 replications and the experiment was repeated.

2.4 Data Analysis

The experiments were analyzed using the software R.[28] Absorption and translocation over time were analyzed using a rectangular hyperbolic model.[29] 2,4-D metabolism and 2,4-D dose response with malathion were analyzed using a three-parameter log-logistic model.[30] Malathion effect on 2,4-D metabolism was analyzed using a factorial ANOVA in R and contrast comparisons were adjusted by the Tukey method.

3 RESULTS

3.1 [^{14}C] 2,4-D Absorption and Translocation

To investigate the 2,4-D resistance mechanisms in *A. tuberculatus*, we first determined if reduced absorption or translocation of [^{14}C] 2,4-D contributed to resistance. There were no differences in the amount of [^{14}C] 2,4-D absorbed between 2,4-D-resistant or -susceptible plants at all harvest times (Figure 1A, Supporting Information Tables 1 and 2). No difference was found in A_{max} (maximum absorption) between populations (S: 73% ±4 and R: 73% ±4) (Figure 1A), or in t_{90} (time in h for 90% of maximum absorption) between populations (S: 43 h ±4 and R: 33 h ±7).

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2,4-D is a systemic herbicide that translocates via xylem and phloem to other parts of the plant following absorption. Translocation was similar between resistant and susceptible plants through 96 HAT (Figure 1B, Supporting Information Tables 1 and 2). Although the experiment was conducted over a reasonable time course of 192 h, 2,4-D translocation in resistant plants did not reach an asymptote by the last time point. The T_{192} value (translocation at 192 HAT) was higher in resistant plants (42 h ±9) than in susceptible plants (23 h ±6). This suggests 2,4-D translocation in susceptible plants is self-limiting beyond 96 HAT when plant death occurs, while 2,4-D translocation continues in resistant plants. Phosphor images confirmed no differences in translocation between the two populations through 96 HAT (Figure 1C). Therefore, differences in 2,4-D absorption or translocation do not contribute substantially to 2,4-D resistance in this *A. tuberculatus* population.

3.2 [^{14}C] 2,4-D Metabolism

To determine if 2,4-D metabolism was a factor in the resistance mechanism of this *A. tuberculatus* population, we measured how much [^{14}C] 2,4-D was metabolized over time. The parent compound of [^{14}C] 2,4-D resolved at peak retention time (RT) of about 12.5 min by reverse-phase HPLC with no other peaks observed (data not shown). This indicates that peaks at other retention times observed in plant lysates are products derived from 2,4-D metabolism (Figures 2A, B). At 264 HAT, a large amount of 2,4-D was detected and just one main metabolite was produced in susceptible plants (metabolite 1), at RT of 10.40 min (Figure 2A). In resistant plants, a small 2,4-D peak was detected and another main metabolite was produced at RT of 8 min (metabolite 4, Figure 2B). Additional metabolites were also detected, including metabolite 1 also found in susceptible plants, metabolite 2 (RT = 9.5 min), metabolite 3 (RT = 8.7 min), metabolite 5 (RT = 7 min), and metabolite 6 (RT = 2 min) (Figure 2B). Analyzing

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metabolism over time using a log-logistic model (Figure 2C, Supporting Information Table 1) showed that resistant plants had a 2,4-D half-life (time to reach 50% 2,4-D metabolism) of 22 h ±4, five times faster than susceptible plants (105 h ±7). The time to reach 70% 2,4-D metabolism in resistant plants was 54 h ±4, and 307 h ±36 for susceptible plants. From these results, it is evident that the resistant *A. tuberculatus* plants rapidly metabolize 2,4-D (Supporting Information Table 3).

3.3 Malathion Effects on 2,4-D Resistance and Metabolism

To test the hypothesis that enhanced 2,4-D metabolism was conferred by cytochrome P450, the known cytochrome P450-inhibitor malathion was tested. The 2,4-D dose required to reduce growth by 50% (GR_{50}) in resistant plants in the absence of malathion was 176 g ha^{-1} ±37, eight times higher than the GR_{50} for susceptible plants (22 g ha^{-1} ±5). Pre-treatment with malathion followed by 2,4-D dose response resulted in the resistant population having a 7-fold reduction in GR_{50} compared to no pre-treatment and a similar 2,4-D response as the susceptible population (Figure 3A, Supporting Information Table 1). With malathion pre-treatment, the GR_{50} for resistant plants was 27 g ha^{-1} ±10, similar to the GR_{50} for susceptible plants following malathion pre-treatment (22 g ha^{-1} ±3).

To investigate whether malathion affected 2,4-D metabolism, malathion treated and untreated plants were treated with [^{14}C] 2,4-D and harvested 264 HAT. Malathion reduced 2,4-D metabolism in both resistant and susceptible populations (Figure 3B). With 2,4-D treatment only, susceptible plants had 25% of the parent 2,4-D remaining at 264 HAT while resistant plants had 7% parent 2,4-D remaining. Following malathion treatment, the resistant and susceptible populations had similar amounts (73% and 74%, respectively) of parent 2,4-D remaining at 264 HAT (Figure 3B, Supporting Information Table 1).

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4 DISCUSSION

4.1 Metabolism of 2,4-D primarily contributes to 2,4-D resistance in *A. tuberculatus*

Auxinic herbicides were the first chemical family of selective herbicides to be discovered and are the most widely used selective herbicides. The phenoxy herbicide 2,4-D is effective in controlling a number of broadleaf weeds including *A. tuberculatus*. Herbicide resistance mechanisms have been categorized into two types, a) non-target-site, involving decreased absorption, translocation and/or enhanced herbicide metabolism, and b) target-site, resulting from mutations in the target gene or increased levels of the target protein by gene amplification or transcriptional upregulation.[31] Previous research found that auxinic herbicide resistance in wild mustard (*Sinapis arvensis*),[32] false cleavers (*Galium spurium*),[33] kochia (*Kochia scoparia*),[34] and yellow starthistle (*Centaurea solstitialis*)[35,36] was not due to differences in herbicide absorption, translocation and/or metabolism and, by deduction, might be due to other mechanisms, such as altered target site. A different dicamba-resistant *K. scoparia* population was found to have reduced dicamba translocation.[37]

In this research, 2,4-D resistance was investigated by determining [^{14}C] 2,4-D uptake, translocation, and metabolism in resistant and susceptible *A. tuberculatus* populations from NE. Our results indicate that 2,4-D absorption and translocation were similar between resistant and susceptible *A. tuberculatus*, and therefore do not appear to contribute to resistance. Previously, a similar amount of total 2,4-D absorption and translocation was reported in leafy spurge (*Euphorbia esula*) and cucumber (*Cucumis sativus*).[38,39] However, in 2,4-D susceptible ground ivy (*Glechoma hederacea*), 37% more 2,4-D was absorbed than in resistant plants.[40] In a Jimsonweed (*Datura stramonium*) population susceptible to 2,4-D, about 70% of the absorbed 2,4-D was translocated within the plant.[41] Reduced MCPA (phenoxy herbicide) translocation

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was found in resistant hemp-nettle (*Galeopsis tetrahit*) compared to susceptible.[42] Recently, reduced 2,4-D translocation was found to confer resistance in a wild radish (*Raphanus raphanistrum*) population.[43] However, in another wild radish population resistant to MCPA, it was found that the resistant plants translocated MCPA more rapidly to roots than did susceptible plants, and also less [^{14}C] MCPA (as % applied) was recovered in resistant plants than in susceptible plants at 48 and 72 HAT.[44] In that study, [^{14}C] MCPA was translocated to the roots, but in *A. tuberculatus*, most of the translocated radioactivity was found in the foliage and very little in the roots. The higher translocation observed in resistant *A. tuberculatus* at 264 HAT may be related to the possible greater mobility of 2,4-D metabolites than parent 2,4-D, as well as the possibility of self-limiting translocation in susceptible plants once plant death occurs.

Our results show that enhanced 2,4-D metabolism contributes to resistance in the *A. tuberculatus* population from NE. The susceptible plants had higher parent [^{14}C] 2,4-D remaining at all time points. The model of 2,4-D metabolism over time showed that resistant plants metabolized 2,4-D seven times faster than did susceptible plants. Previously, 2,4-D-susceptible hemp dogbane (*Apocynum cannabinum*) was found to metabolize only 48% of the herbicide at 12 d after application.[45] *Euphorbia esula* plants susceptible to 2,4-D contained 85% of the parent [^{14}C] 2,4-D at 72 HAT.[38] One study reported elevated 2,4-D metabolism in less-susceptible wild cucumber when compared to more-susceptible cultivated cucumber.[39] An MCPA-resistant *G. tetrahit* population had increased MCPA metabolism compared to a susceptible population.[42] The bacterial aryloxyalkanoate dioxygenase transformed in 2,4-D resistant crops show that rapid 2,4-D metabolism can confer robust 2,4-D resistance.[3] Collectively these results suggest that if enough 2,4-D is metabolized in *A. tuberculatus* from 24-

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48 HAT, the enhanced metabolism will enable the resistant plant to survive short-term 2,4-D induced toxicity and continue to grow.

Auxinic herbicide selectivity in crops is primarily dependent on plant metabolism of these herbicides. Metabolic detoxification of 2,4-D typically occurs through side-chain cleavage, or ring hydroxylation followed by glucose conjugation. Tolerant plants can convert the parent biologically active molecule to more polar and insoluble residues.[46] Sensitive species can sometimes metabolize 2,4-D faster than tolerant species, however, the main metabolites formed in sensitive species are reversible conjugates that can rapidly convert back to the biologically active, parent compound.[47] The metabolites produced by tolerant species are generally more stable and irreversible.[47] In auxinic herbicide-tolerant monocots, the formation of stable metabolites via phenyl and heterocyclic ring hydroxylation followed by subsequent sequestration of the non-biologically active compounds has been reported.[48]

The specific reactions involved in 2,4-D detoxification in our resistant population need to be investigated. One main metabolite was produced in susceptible plants while resistant plants produced the same metabolite with several additional metabolites. The structures of these metabolites have not yet been identified, but this information would help determine the biochemical steps involved in the enhanced 2,4-D metabolism in resistant plants. In our malathion experiments, we showed that this cytochrome P450 inhibitor reduced 2,4-D metabolism at 264 HAT in resistant plants and reversed 2,4-D resistance in a whole-plant dose response. Cytochrome P450s are versatile enzymes involved in phase I of herbicide metabolism including ring hydroxylation, and plants have a high diversity of cytochrome P450 gene families that are able to metabolize natural and xenobiotic compounds.[49,50] Many weed species have been reported with enhanced metabolic resistance mediated by cytochrome P450s to various herbicide

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modes of action including ALS, acetyl Co-A carboxylase (ACCase), photosystem II, and HPPD.[51,52] Metabolic resistance in *A. tuberculatus* has been previously reported for ALS,[53] photosystem II[54] and HPPD[54,55] herbicides, with different cytochrome P450 genes likely conferring HPPD resistance in different populations.[54,56] 2,4-D has been reported as an inducer of cytochrome P450 activity in plants both *in vitro*[57,58] and *in vivo*,[59] including the induction of demethylation and ring-methyl hydroxylation of chlortoluron in tobacco (*Nicotiana tabacum*) cells.[58] More recent studies showed that ACCase-inhibitor-susceptible *Lolium* plants pre-treated with 2,4-D had induction of cytochrome P450 transcripts[60] and higher rates of diclofop-methyl metabolism, which was reversed after malathion treatment.[59]

In conclusion, these results clearly demonstrate 2,4-D metabolism as a contributing factor for 2,4-D resistance in *A. tuberculatus*. Reversal of resistance and reduced 2,4-D metabolism following treatment with the cytochrome P450 inhibitor malathion indicate that one or more cytochrome P450 genes mediate this enhanced 2,4-D metabolism. Metabolism-based herbicide resistance is a particular challenge as it may confer complex and sometimes unpredictable cross-resistance to current and yet-to-be-discovered herbicides.[51,61]

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Figure Legends

Figure 1. [^{14}C]-labeled 2,4-D absorption and translocation in resistant (R) and susceptible (S) *A. tuberculatus* over a 96 h time course (conducted at Colorado State University). A) 2,4-D absorption as percentage of applied radioactivity. B) 2,4-D translocation as percentage of absorbed radioactivity. C) Phosphor images showing 2,4-D translocation over time with the corresponding plant color image to the left of the phosphor image.

Figure 2. [^{14}C]-labeled 2,4-D metabolism in resistant and susceptible *A. tuberculatus* (conducted at Colorado State University). A) Susceptible and B) resistant HPLC chromatograms of [^{14}C] 2,4-D metabolism at 264 HAT (radioactive units in Bq vs retention time in min), with different metabolites numbered in order of their respective retention times. C) Non-linear regression of 2,4-D metabolism at different time points after herbicide treatment with dashed lines indicating 2,4-D half-life.

Figure 3. Malathion reverses 2,4-D resistance and metabolism in resistant (R) and susceptible (S) *A. tuberculatus*. A) Dry weight dose response of R and S with and without malathion pre-treatment (Mal), 28 d after 2,4-D application with dashed lines indicating GR_{50} (2,4-D dose required to reduce biomass by 50%). B) HPLC chromatograms of [^{14}C] 2,4-D metabolism (radioactive units in Bq vs retention time in min) at 264 h after 2,4-D application in R and S with and without malathion pre-treatment. Percentage indicated above 2,4-D retention time (13.4 min) represents the mean parent [^{14}C] 2,4-D measured in all replicates. Other peaks represent 2,4-D metabolites. Letters represent significant differences between R and S (upper case) or between malathion treatments (lower case) with Tukey's test (n=6; $\alpha = 0.5$).

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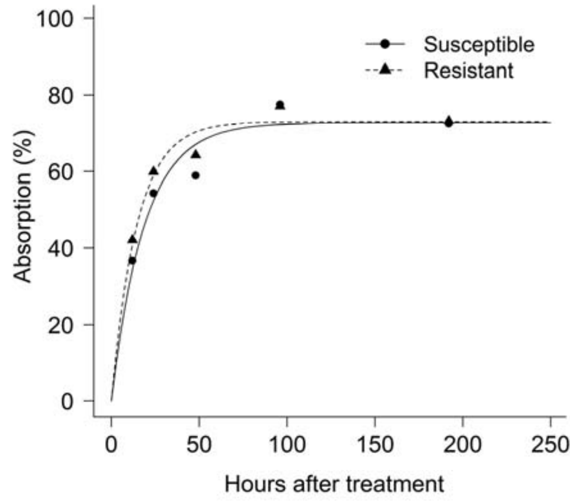
A



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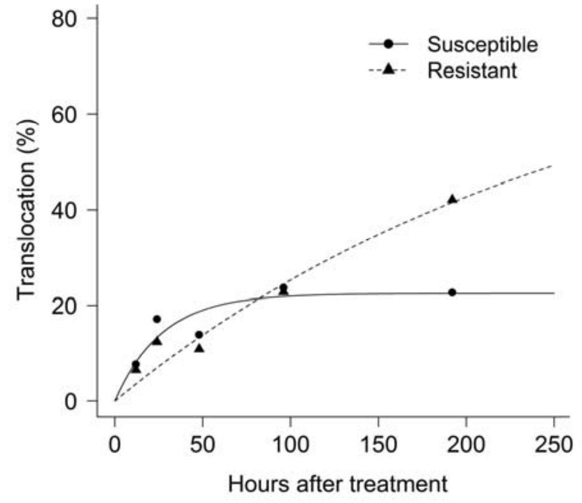

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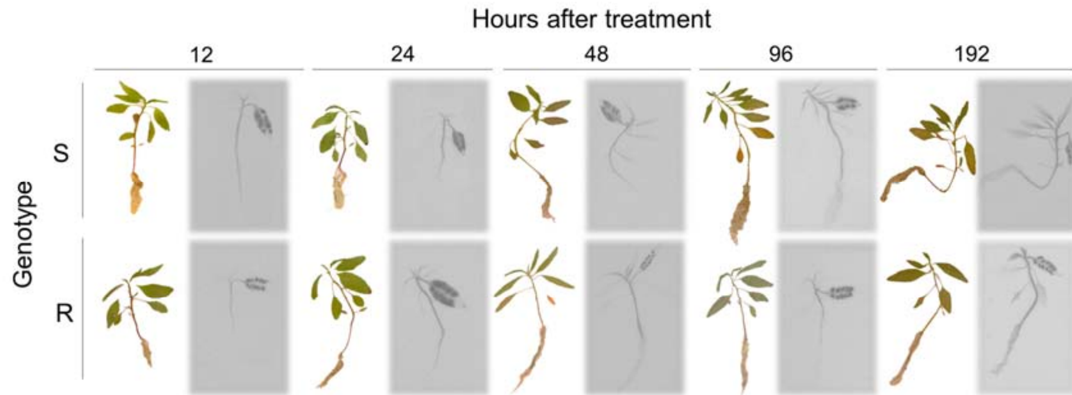

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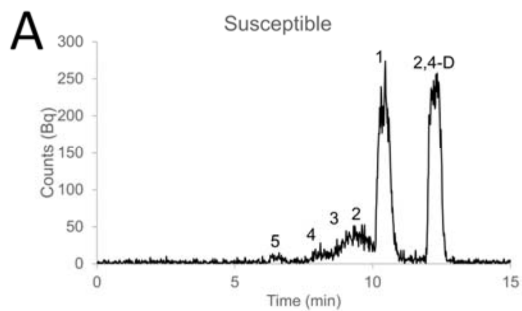

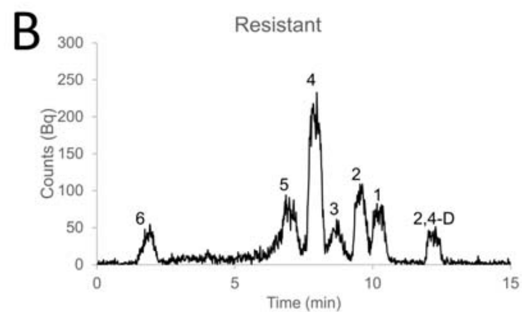

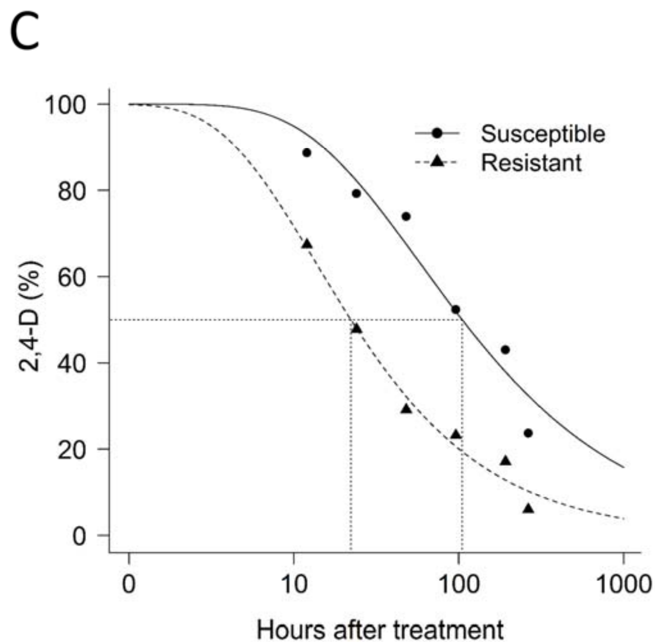

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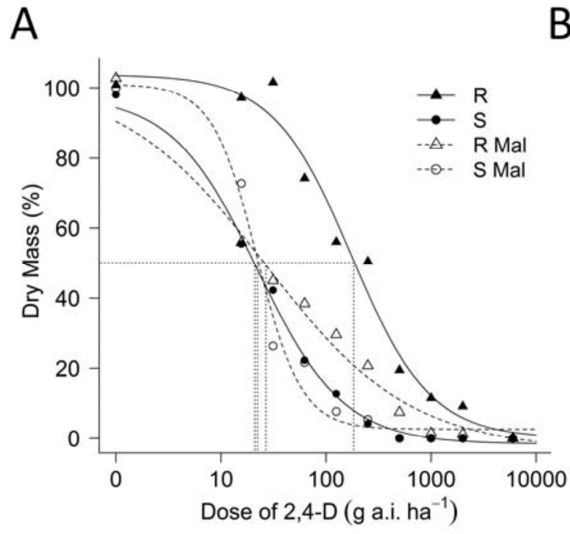

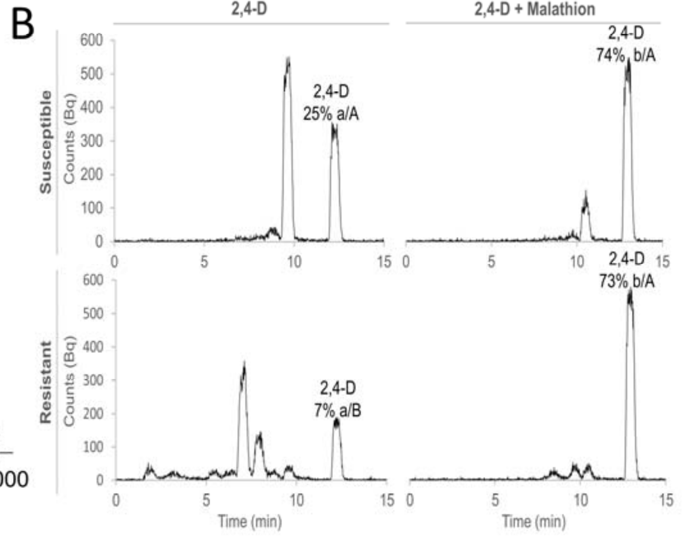

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SUPPORTING INFORMATION

Metabolism of 2,4-dichlorophenoxyacetic acid contributes to resistance in a common waterhemp (*Amaranthus tuberculatus*) population

Results

Supporting Information Table 1. Equation parameters for [^{14}C] 2,4-D absorption, translocation, and metabolism.

Figure	Population	Equation
1A, absorption	Susceptible	f(x) = (72.6907(x))/(0.11*43.2083+x)
	Resistant	f(x) = (72.9682 (x))/(0.11*33.4752+x)
1B, translocation	Susceptible	f(x) = (22.4823(x))/(0.11*61.3814+x)
	Resistant	f(x) = (81.0326 (x))/(0.11*614.8625+x)
2C, metabolism	Susceptible	f(x) = (100) exp(− exp(-0.618446 (log(x) – 58.015172)))
	Resistant	f(x) = (100) exp(− exp(-0.749272 (log(x) − 13.595200)))
3A, metabolism	Susceptible, - malathion	f(x) = ((3.05020)/ (1 + exp(1.13179(log(x) − log(21.74716))))
	Resistant, - malathion	f(x) = ((3.23644)/ (1 + exp(1.16502(log(x) − log(176.48644))))
	Susceptible, + malathion	f(x) = ((3.15715)/ (1 + exp(1.78556(log(x) − log(22.74036))))
	Resistant, + malathion	f(x) = ((3.18549)/ (1 + exp(0.69062(log(x) − log(24.42846))))

Supporting Information Materials and Methods

[^{14}C] 2,4-D Absorption and Translocation

In an first experiment at Kansas State University (KSU), 2,4-D-resistant and susceptible *A. tuberculatus* were grown in a greenhouse (25/20ºC day/night temperature, 15/9 h day/night photoperiod). When the seedlings reached 5-6 cm tall, they were transferred to growth chambers maintained at 32.5/22.5 ºC, 15/9 h photoperiod, and 60-70% relative humidity. Light in the growth chamber was provided by fluorescent bulbs delivering 550 µmol m^{-2} s^{-1} photon flux at plant canopy level. Plants were watered as needed both under greenhouse and growth chamber conditions. Ten to 12 cm tall plants were treated with four × 2.5 µl (3.33 kBq) droplets of [^{14}C] 2,4-D on the adaxial surface of a fourth or fifth youngest leaf, which was marked with a black permanent marker. Unlabeled 2,4-D was added to the radioactive solution to obtain the field labeled rate of 280 g ha^{-1} in a carrier volume of 187 L ha^{-1}. The adjuvants crop oil concentrate (COC, Agridex, Helena Holding Co., Wilmington, DE) and ammonium sulfate (AMS, Liquid N-PaK; Agriliance, LLC, Inver Grove Heights, MN) were added at 1% v/v and 0.85% v/v, respectively, to maximize adherence of herbicide solution to the leaf surface. The treated plants were returned to the same growth chamber. Plants were harvested at 6, 24, 48 and 72 h after treatment (HAT) and dissected into the tissue of treated leaf (TL), above the treated leaf (ATL), below the treated leaf (BTL), and roots (R). Treated leaves were rinsed for approximately 60 sec with 5 ml wash solution containing 10% methanol and 0.05% Tween™ to remove any herbicide that was not absorbed. Liquid scintillation spectrometry (LSS; Tricarb 2100 TR Liquid Scintillation Analyzer; Packard Instrument Co., Meriden, CT) measured the amount of radioactivity in the leaf rinsate. The harvested samples were wrapped in a single layer of tissue paper and dried at 60°C for 16 h. Subsequently, the plant samples were combusted using a

biological oxidizer (OX-501, RJ Harvey Instrument, Tappan, NY) and radioactivity was determined via LSS. Total 2,4-D absorption was determined by the following equation: % absorption = (total radioactivity applied – radioactivity recovered in wash solution) × 100 / total radioactivity applied. Herbicide translocation to each plant tissue was determined by the following equation: % absorbed = (radioactivity oxidized in plant tissue/total radioactivity absorbed) × 100. Total translocation was the sum of radioactivity recovered in ATL, BTL, and R.

[^{14}C] Metabolism

In an experiment at KSU, 2,4-D-resistant and –susceptible common plants were grown as described previously for [^{14}C] 2,4-D absorption and translocation experiments. Ten to 12 cm tall plants were treated with [^{14}C] 2,4-D (3.99 kBq) as ten by 1µL droplets on the adaxial surface of fully expanded fourth and fifth youngest leaves. To remove any unabsorbed herbicide, the treated leaf was harvested and subsequently rinsed with 5% Tween™ solution at 24, 48, and 72 HAT. All above ground plant tissue was immediately frozen in liquid nitrogen to prevent ongoing metabolism and then homogenized with mortar and pestle. [^{14}C] 2,4-D and its metabolites were extracted as described[27] with minor modifications. Samples were centrifuged at 5,000×g for 10 min. Supernatants were extracted and concentrated for 2-3 h at 45°C until reaching an approximate final volume of 500 µl (Centrivap, Labconoco, Kansas City, MO). The 500 µl extract samples were transferred to 1.5 ml microcentrifuge tubes and then centrifuged 10 min at 10,000×g. Total radioactivity per sample was measured via LSS. Samples were then normalized to 6,000 dpm using acetonitrile:water (50:50, v/v) prior to high-performance liquid chromatography (HPLC).

Total extractable radioactivity in 50 µL was resolved into parent [^{14}C] 2,4-D and its metabolites by reverse-phase HPLC (Beckman Coulter, System Gold, Brea, CA) following the protocol optimized previously in our laboratory[27]. Reverse-phase HPLC was performed with a Zorbax SB-C18 column (4.6 × 250 mm, 5-µm particle size; Agilent Technologies) at a flow rate of 1 mL min^{-1}. The radioactivity in the sample was measured using radio flow detector LB 5009 (Berthold Technologies). The metabolism experiment had three replicates for each treatment and the experiment was repeated. As the parent [^{14}C] 2,4-D had a retention time of 11.6 min in the KSU experiment, the radioactivity measured at this retention time was considered to be non-metabolized [^{14}C] 2,4-D. The percent non-metabolized [^{14}C] 2,4-D was calculated as the radioactivity measured at 11.6 min compared to total amount recovered.

Data Analysis

The experiments conducted at KSU were in randomized complete blocks and a single plant represented an experimental unit. Absorption and translocation experiments included four replications and experiments were conducted twice. The metabolism studies included three replications and were conducted twice. All data were analyzed using the PROC GLIMMIX procedure of SAS (SAS Institute Inc., Cary, NC 27513) for generalized linear mixed model analysis to incorporate normally distributed random effects. Variances were homogenous among individual runs within each experiment and thus runs were combined for analysis and presentation. Treatment means were separated by Fisher's protected least significant difference at $P \leq 0.05$ level of significance.

Supporting Information Table 2. Absorption (percentage of radioactivity applied) and translocation (percentage of absorbed radioactivity) of [^{14}C]-2,4-D in 2,4-D-resistant (R) and –susceptible (S) *A. tuberculatus*. Data are means with standard errors in parentheses from experiment conducted at Kansas State University. Means followed by different letters indicate significant differences.

		Time after treatment				
Plant part	**Biotype**	**6 h**	**24 h**	**48 h**	**72 h**	**96 h**
		^{14}C 2,4-D (as % applied)				
Leaf rinse	R	63.22 (2.73) a	47.65 (3.04) a	51.02 (2.59) a	49.39 (3.57) a	43.87 (2.21) a
	S	59.51 (0.84) a	45.59 (3.55) a	48.69 (2.89) a	49.17 (3.30) a	41.88 (2.95) a
Total absorbed	R	36.77 (2.73) a	52.34 (3.04) a	48.97 (2.59) a	50.61 (3.57) a	56.12 (2.21) a
	S	40.49 (2.44) a	54.40 (3.55) a	51.30 (2.89) a	50.82 (3.30) a	58.11 (2.95) a
		^{14}C 2,4-D recovered in plant (as % absorbed)				
Treated leaf (TL)	R	96.40 (1.19) a	89.96 (7.17) a	95.02 (1.07) a	93.28 (1.61) a	95.60 (0.66) a
	S	96.38 (0.11) a	87.98 (7.20) a	91.70 (2.60) a	90.11 (1.77) a	92.46 (2.35) a
Shoot above (ATL)	R	0.59 (0.19) a	0.85 (0.33) a	0.91 (0.22) a	0.67 (0.12) a	0.39 (0.06) a
	S	0.58 (0.71) a	0.81 (0.41) a	2.43 (1.95) a	1.15 (0.43) a	0.78 (0.26) a
Shoot below (BTL)	R	2.43 (0.93) a	3.64 (2.22) a	2.55 (0.63) a	4.54 (1.39) a	2.26 (0.45) a
	S	2.73 (0.07) a	9.78 (6.18) a	4.50 (0.88) a	6.50 (1.09) a	4.82 (1.86) a
Roots (BG)	R	0.55 (0.15) b	5.53 (4.68) a	1.51 (0.41) a	1.50 (0.37) b	1.73 (0.33) b
	S	0.41 (0.07) a	1.40 (0.64) b	1.35 (0.51) b	2.22 (0.73) a	1.93 (0.30) a
Total translocated	R	3.59 (1.20) a	10.03 (7.17) a	4.98 (1.07) a	6.72 (1.62) a	4.39 (0.67) a
(ATL+BTL+BG)	S	3.62 (0.84) a	12.01 (7.20) a	8.30 (2.60) a	9.89 (1.78) a	7.53 (2.36) a

Supporting Information Table 3. Least square means and ANOVA of percent parent compound [^{14}C] 2,4-D remaining in resistant and susceptible *A. tuberculatus* populations (P) at three harvest (H) timings from experiment conducted at Kansas State University.

	Parent Compound [^{14}C] 2,4-D* (%)	
Harvest	Resistant	Susceptible
24 HAT	47.8	84.3
48 HAT	29.4	57.2
72 HAT	33.6	53.3
ANOVA		
P	<0.0001	
H	0.0004	
P by H	0.3609	

*Analysis of variance using PROC GLIMMIX in SAS 2013 using Fisher's Protected LSD at P ≤ 0.05 level of significance. Values reflect three replications and two runs. Each plant received 3.98 kBq of radiation.