Rapid metabolism increases the level of 2,4-D resistance at high temperature in common waterhemp (*Amaranthus tuberculatus*)

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Common waterhemp emerges throughout the crop growing season in the Midwestern United States, and as a result, the seedlings are exposed to a wide range of temperature regimes. Typically, 2,4-D is used in the Midwest to control winter annual broad-leaf weeds before planting soybean and in an early post-emergence application in corn and sorghum; however, the evolution of 2,4-D-resistant common waterhemp in several Midwestern states may limit the use of 2,4-D for controlling this problem weed. Moreover, temperature is one of the crucial factors affecting weed control efficacy of 2,4-D. This research investigated the effect of temperature on efficacy of 2,4-D to control 2,4-D susceptible (WHS) and -resistant (WHR) common waterhemp. Dose-response of WHS and WHR to 2,4-D was assessed at two temperature regimes, high (HT; 34/20 °C, d/n) and low (LT; 24/10 °C, d/n). Whole plant dose response study indicated an increased level of 2,4-D resistance in WHR at HT compared to LT. Additional investigation of the physiological mechanism of this response indicated that both WHS and WHR common waterhemp plants rapidly metabolized 14C 2,4-D at HT compared to LT. In conclusion, a rapid metabolism of 2,4-D conferred increased level of resistance to 2,4-D in WHR at HT. Therefore, application of 2,4-D when temperatures are cooler can improve control of 2,4-D resistant common waterhemp.

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WHS and WHR control at a high (HT; 34/20 °C, d/n) and low (LT; 24/10 °C, d/n) temperature regimes, and (2) understanding the effect of temperature on efficacy of 2,4-D as a post-emergence option will help to better facilitate control of 2,4-D-resistant and susceptible common waterhemp. Due to reduced absorption of glyphosate and reduced translocation of dicamba, scientific literature is not existing for 2,4-D-resistant common waterhemp. Therefore, there was no significant difference between GR50 of WHR population to GR50 of the WHS population. Values in parenthesis are standard error of mean.

Table 1. Regression parameters estimated from the whole-plant 2,4-D dose-response study based on dry shoot biomass of 2,4-D–susceptible (WHS) and –resistant (WHR) common waterhemp grown under low (24/10 °C, d/n) and high (34/20 °C, d/n) temperature regimes at 4 weeks after treatment (WAT). ‘Data combined from two runs.’ GR50 is the effective 2,4-D doses (g ae ha⁻¹) required for 50% reduction in shoot dry biomass. ‘RI’ is calculated as a ratio of GR50 of the WHR population to GR50 of the WHS population. Values in parenthesis are standard error of mean.

| Population | Temperature (°C) | Effective herbicide dose (GR50 g ae ha⁻¹) | Resistance Index (RI) | Regression parameters |
|------------|-----------------|------------------------------------------|-----------------------|-----------------------|
|            |                 | B                                         | D                     |                       |
| WHS        | 24/10           | 107 (±0.26)                              | —                     | 0.68 (±0.14)          | 99.88 (±6.00)         |
|            | 34/20           | 178 (±0.43)                              | —                     | 0.76 (±0.11)          | 101.27 (±5.80)        |
| WHR        | 24/10           | 1001 (±237)                              | 9.35                   | 0.81 (±0.14)          | 100.39 (±5.95)        |
|            | 34/20           | 3696 (±1138)                             | 20.76                  | 0.65 (±0.16)          | 100.57 (±5.58)        |

Results
2,4-D dose-response experiment. WHS and WHR exhibited varying response to 2,4-D at HT or LT regime (Fig. 1). At 4WAT, the amount of 2,4-D required to reduce 50% (GR50) growth of WHS and WHR plants grown at HT regime were 178 and 3,696 g ae ha⁻¹ and while at LT regime were 107 and 1,001 g ae ha⁻¹, respectively (Table 1). Thus, the resistance indices of WHR relative to WHS grown at HT and LT regimes were ~20 and ~10, respectively, suggesting that WHR common waterhemp showed increased level of resistance to 2,4-D at HT compared to LT (Fig. 1, Table 1). “CompParm” function in R indicated that there is significant difference between GR50 of WHR at HT and LT (p < 0.05). WHR and WHS at HT (p < 0.001). However, there was no significant difference between GR50 of WHS at HT and LT. This suggests reduction in efficacy of 2,4-D at LT to control 2,4-D-resistant common waterhemp (Fig. 1).

The test for ‘lack of fit’ in ‘drc’ was non-significant (p = 0.88), suggesting that the data fitted the regression model reasonably. Root means square error (RMSE) values of the 2,4-D dose-response experiments conducted at HT and LT ranged from 1.82 to 2.48 for WHS and 2.65 to 2.04 for WHS respectively, indicating a good fit.

14C 2,4-D absorption and translocation experiment. Regression analysis of 14C 2,4-D absorption indicated that temperature did not affect the absorption or translocation of 14C 2,4-D in both WHS and WHR and there was no significant difference between Amax (upper limit of absorption) and Am (the time required to achieve 90% of maximum absorption) of WHR and WHS at HT and LT conditions. Amax for WHS and WHR at HT and LT regimes were 96.31 (±3.70), 92.73 (±3.61), and 93.43 (±2.54), and 95.35 (±3.16) %, respectively (Table 2).
Moreover, $A_{90}$ was also similar in WHS and WHR plants at HT or LT regimes i.e., 18 ($\pm$6.19), 13 ($\pm$7.38), 16.43 ($\pm$5.17), and 22.12 ($\pm$7.61) hours, respectively (Table 2). Similarly, there was no significant difference between $T_{\text{max}}$ (upper limit of translocation) and $T_{90}$ (the time required to achieve 90% of maximum translocation) between WHS and WHR at two temperature regimes, which indicated that temperature regimes did not affect $\text{14C 2,4-D}$ translocation. The predicted $T_{\text{max}}$ for WHS and WHR at HT and LT regimes were 75.69 ($\pm$14.39), 79.18 ($\pm$14.03) and 70.83 ($\pm$14.39), and 73.78 ($\pm$18.92) %, respectively (Table 2). The time required to achieve 90% of the maximum translocation of 2,4-D in WHS and WHR plants were 111.63 ($\pm$55.07), 119.73 ($\pm$70.20) and 113.12 ($\pm$77.17), 120.59 ($\pm$94.74) hours, respectively, at HT and LT regimes (Table 2).

**14C 2,4-D metabolism experiment.** The HPLC chromatographs indicated that the retention time of the parent $^{14}$C 2,4-D (used as standard) was 11.96 min (Fig. 2). Peaks of parent 2,4-D were much taller in WHR at LT compared to HT at 24 and 72 HAT. However, such difference was not observed at 6 HAT in WHR plants (Fig. 3b). At 6 HAT, the mean 2,4-D retention by WHR and WHS common waterhemp at HT and LT temperature regimes was 69.3, 69.3%, and 85.1, 95.3%, respectively (Fig. 3a,b). Twenty-four HAT, WHR plants retained 20.2 and 47.7% of parent 2,4-D at HT (Figs 2d and 3b) and LT (Figs 2c, 3b), respectively. Whereas, WHS retained 82.3 (Figs 2b and 3a) % at LT, respectively. This validates that, metabolism of 2,4-D plays a key role in bestowing 2,4-D resistance in WHR (Fig. 2). More importantly, this indicates that at 24 HAT, WHR plants grown at LT retained approximately 27% more parent 2,4-D than at HT (Figs 2c,d and 3b). This indicates rapid metabolism of 2,4-D in WHR plants grown at HT compared to LT. Also, at 72 HAT, the WHR plants grown at HT conditions metabolized close to 100% of the parent 2,4-D while those at LT still retained 9.4% (Fig. 3b). At 72 HAT the WHS plants retained 33.7, 54.5% of parent 2,4-D at HT and LT conditions, respectively (Fig. 3a). Overall, the rate of 2,4-D metabolism increased both in WHR and WHS at HT (Fig. 3a,b).

The two-way analysis of parent 2,4-D retained in WHR followed by mean comparison using LSD ($p = 0.05$) suggested that there is a significant difference in % parent 2,4-D present in WHR at 24 HAT (Fig. 3b) with more 2,4-D being retained in plants grown at LT. In case of WHS plants, such difference was observed at 72 HAT (Fig. 3a) with more 2,4-D retained at LT compared to HT.

**Figure 1.** Whole-plant 2,4-D dose-response of 2,4-D susceptible (WHS) and -resistant common waterhemp (WHR) at low (LT; 24/10 °C, d/n) and high (HT; 34/20 °C, d/n) temperature regimes based on dry shoot biomass at 4 weeks after treatment (WAT).

**Table 2.** Regression parameter estimates of $^{14}$C 2,4-D absorption and translocation of 2,4-D- susceptible (WHS) and -resistant (WHR) common waterhemp at low (24/10 °C, d/n) and high (34/20 °C, d/n) temperature regimes using rectangular hyperbola model. $^a$Data combined from two runs. $^b$A$_{\text{max}}$ and T$_{\text{max}}$ is the maximum absorption or translocation (%), A$_{90}$ or T$_{90}$ is the time (h) required to achieve 90% of the maximum absorption or translocation. $^c$Values in parenthesis are standard error of mean.
Discussions

The time of emergence of common waterhemp under field conditions depends on various factors including, soil temperature, moisture, and seed dormancy. Especially, in the Midwestern United States, common waterhemp emergence occurs over a wider time frame compared to other summer annual weed species. The average diurnal temperatures in May and July, the two major seasons for waterhemp cohort emergence, are around 24/10 °C and 34/20 °C in Kansas (Fig. 4). The dose-response study results demonstrated reduced efficacy of 2,4-D at HT (34/20 °C) compared to LT (24/10 °C) for controlling both WHS and WHR common waterhemp. In contrast, Ganie et al. reported improved efficacy of 2,4-D or glyphosate at HT (29/17 °C) compared with LT (20/11 °C) for common and giant ragweed control regardless of susceptibility or resistance to glyphosate. Godar et al. reported reduced efficacy of mesotrione for Palmer amaranth (Amaranthus palmeri) control at high (40/30 °C) compared to low (25/15 °C) temperature due to reduced translocation coupled with rapid metabolism of mesotrione and increased 4-hydroxyphenylpyruvate dioxygenase (HPPD)-gene expression. However, as previously reported by Figueiredo et al., the data from this study also showed no difference in 2,4-D absorption or translocation between WHR and WHS (Table 2). Previous studies have shown that 2,4-D absorption can range from 10–99% depending on several factors such as environment, weed species and other application factors. Similar to our findings, Coetzer et al. reported no effect of temperature on glufosinate absorption in Palmer amaranth.
High temperature increased the rate of metabolism of 2,4-D both in WHR and WHS common waterhemp. Similar to these findings, Johnson and Young\cite{Johnson2006}, reported a 6–7-fold higher susceptibility of common waterhemp to mesotrione at 18°C compared to 32°C. Likewise, Olsen et al.\cite{Olsen2008} reported decreased metabolism of MON 37500 in several grass weeds (Aegilops cylindrica, Avena fatua, Bromus tectorum) grown at cool air temperature. Galleher et al.\cite{Galleher2005} observed rapid metabolism of primisulfuron and nicosulfuron in broadleaf signalgrass (Brachiaria platyphylla) at high (30/20°C) compared to low (20/10°C) temperature.

The auxinic herbicide-tolerant monocotyledonous weeds are known to metabolize 2,4-D via ring hydroxylation mediated by cytochrome P-450 monooxygenases, an enzyme family predominantly involved in metabolizing xenobiotics in plants\cite{Lamie2006, Merkle2006}. A possible involvement of these enzymes in 2,4-D degradation has been documented in many dicotyledonous weeds, resistant to this herbicide. For example, cytochrome P-450 mediated 2,4-D degradation has been reported in 2,4-D-resistant corn poppy\cite{Figueiredo2011}. Figueiredo et al.\cite{Figueiredo2011} reported a 7-fold reduction in GR_{50} of WHR (the same common waterhemp) with pre-treatment of malathion (a cytochrome P-450-inhibitor) followed by 2,4-D compared to plants treated with 2,4-D alone, indicating a possible involvement of cytochrome P-450s in 2,4-D metabolism in common waterhemp. Thus, it is likely that a rapid metabolism of 2,4-D in WHR plants grown at HT is facilitated by increased activity of cytochrome P-450 enzymes. Previously, Viger et al.\cite{Viger2007} reported rapid metabolism of metolachlor at a high temperature (30°C) compared to a low temperature (21°C), which was associated with a five-fold increase in glutathione-S-transferase (GST) activity in corn. Therefore, the possible increased cytochrome P-450 enzyme activity may be an example of common waterhemp adaptation to high temperature stress. Studies have shown that plant response to stress, including abiotic stress can lead to further selection of resistant weed biotypes\cite{Johnson2006}. Hence, application of 2,4-D at the most effective temperature regime is important to control common waterhemp and reduce further selection of 2,4-D resistance.

In conclusion, the results of this research demonstrate that 2,4-D efficacy can be improved at low temperature regime (24/10°C, d/n) to manage common waterhemp. Thus, applying 2,4-D when day temperature is lower than 30°C is desirable for common waterhemp control; however, apart from air temperature other abiotic factors such as light intensity, relative humidity, and plant factors such as leaf orientation also play key role in affecting herbicide efficacy. Our studies were conducted in growth chambers where apart from temperature all other factors were kept constant. This is particularly important to reduce common waterhemp competition and crop yield loss and reduce selection for resistance. In general, the efficacy of auxinic herbicides for controlling dicotyledonous weeds depends on several factors including time of application\cite{Figueiredo2011, Olsen2008, Galleher2005, Johnson2006}. Additionally, efficacy of 2,4-D is species dependent as improved efficacy at HT has been noticed for control of common and giant ragweed. Therefore, further studies are needed to assess the interaction of other abiotic and plant factors that can influence 2,4-D efficacy for controlling common waterhemp.

**Materials and Methods**

**Plant materials and growth conditions.** WHS and WHR common waterhemp from Nebraska, USA were used in this study\cite{Baker2007}. Common waterhemp resistant to 2,4-D (WHR) has been confirmed in a native grass little bluestem (Schizachyrium scoparium) production field in southeastern Nebraska where 2,4-D was applied for over 10 years\cite{Johnson2006}. The susceptible population (WHS) was collected from a soybean field near Auburn, Nebraska\cite{Johnson2006, Baker2007}.

WHS and WHR common waterhemp seeds were germinated in plastic trays (25 × 15 × 2.5 cm) filled with potting mix (Fafard® ultra container potting mix, Sungro Horticulture, Agawam, MA). After emergence, individual seedlings at 2–3 leaf stage were transplanted into plastic pots (6 × 6 × 6 cm) and kept in the greenhouse maintained at 25/20° C day/night (d/n), 15 hours of photoperiod supplemented with 120 μmol m\(^{-2}\) s\(^{-1}\) illumination provided with sodium vapor lamps along with 60 ± 10% relative humidity. At 7 days after transplanting, half of the small and uniform seedlings (4-leaf stage) were transferred in growth chambers set at HT (34/20°C, d/n) and the rest were transferred in a separate growth chamber set at LT (24/10°C, d/n). Temperature regimes were selected based on the average diurnal temperatures during mid-May to mid-June in Kansas, USA\cite{Baker2007}. Incandescent and fluorescent bulbs were used in growth chambers to maintain light level of 750 μmol m\(^{-2}\) s\(^{-1}\) (15/9 hrs, d/n condition) and relative humidity was maintained at 60 ± 10% throughout the study. Plants were watered daily and fertilized once a week after transplanting.

**2,4-D dose-response experiment.** Ten to 12 cm tall WHS and WHR common waterhemp plants grown at HT or LT were treated with several rates of 2,4-D (2,4-D Amine 4, Winfield Solutions, LLC, St. Paul MN, 2440°C) to manage common waterhemp. Thus, applying 2,4-D when day temperature is lower than 30°C is desirable for common waterhemp control; however, apart from air temperature other abiotic factors such as light intensity, relative humidity, and plant factors such as leaf orientation also play key role in affecting herbicide efficacy. Our studies were conducted in growth chambers where apart from temperature all other factors were kept constant. This is particularly important to reduce common waterhemp competition and crop yield loss and reduce selection for resistance. In general, the efficacy of auxinic herbicides for controlling dicotyledonous weeds depends on several factors including time of application\cite{Figueiredo2011, Olsen2008, Galleher2005, Johnson2006}. Additionally, efficacy of 2,4-D is species dependent as improved efficacy at HT has been noticed for control of common and giant ragweed. Therefore, further studies are needed to assess the interaction of other abiotic and plant factors that can influence 2,4-D efficacy for controlling common waterhemp.

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placed in paper bags and dried in an oven at 60 °C for 72 hours (h) to measure dry shoot biomass. Percent dry

2,4-D application. At 4 weeks after treatment (WAT), above-ground biomass from each plant was harvested and

avoid effect of growth chamber on plant response. The dose-response experiments were arranged in a two-way

with four replications and repeated in time. Growth chambers were switched between two experimental runs to

fourth youngest fully expanded leaf was treated with 10-

commercially available 2,4-D (2,4-D Amine 4, Winfield Solutions, LLC, St. Paul MN, USA) to obtain 560 g ae ha

metabolites were extracted with 15 ml of 90% aqueous acetone in a centrifuge tube and preserved at 4 °C for at least

16 hours. After 16 hours, the tubes were centrifuged at 5,000

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to a new centrifuge tube and concentrated at 45°C for 1.5–2 h with a rotary evaporimeter (Centrivap, Labconco, Kansas City, MO). The final volume of the supernatant was maintained around 600 µL and transferred to a 1.5 ml microcentrifuge tube and centrifuged at 10,000 × g for 10 minutes. The radioactivity of the supernatant solution was measured with the liquid scintillation counter and normalized by diluting the samples with 50% acetonitrile (1:1 v/v acetonitrile:water). The final solutions were analyzed using reversed-phase high-performance liquid chromatography (HPLC) (BeckmanCoulter system Gold 126 solvent module, Beckman Coulter Inc., Fullerton, CA, USA) to resolve the solution contents into parent 14C 2,4-D and its metabolites.

Experimental design and statistical analysis. The experiments were arranged in a split-plot design with four replications and repeated in time. Growth chambers were switched between two experimental runs to avoid effect of growth chamber on plant response. The dose-response experiments were arranged in a two-way
factorial combination of temperature regimes (HT and LT) as main factor and herbicide doses for each common waterhemp population as sub-plot factor.

Relative shoot biomass data obtained from the whole plant dose-response study were analyzed using the ‘drc’ package (drc 1.2, Christian Ritz and Jens Streibig, R2.5, Kurt Hornik, online) in R (R statistical software, R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org) as per Knezevic et al. A dose-response regression model was constructed using the three-parameter log-logistic equation.

\[
Y = \left\{ \frac{d}{1 + \exp[b(\log X - \log e)]} \right\}
\]

In equation above, Y is response variable (% reduction in biomass compared to control), b denotes relative slope around e, e is GR50 (effective dose to reduce biomass of the population by 50%) and d is the upper limit of the model. The ratio of GR50 values of WHS and WHR common waterhemp in HT and LT conditions were calculated to determine the level of resistance or the resistance index. Estimated GR50 values were then compared with each other using the “compParm” function in ‘drc’ package in R.

Fitness of the log-logistic regression model used above was assessed through the “Lack-of-fit” test in ‘drc’ using “modelFit” function. Further, root mean square error (RMSE) was calculated to test the goodness of fit of the data. The formula used for RMSE was:

\[
RMSE = \sqrt{\frac{1}{n-1} \sum_{i=1}^{n} (P_i - O_i)^2}
\]

where, n is the number of observations and Oi and Pi are the observed and predicted value of the observations respectively.

Absorption, translocation, and metabolism experiments, treatments were arranged in a two-way factorial combination with temperature regime (HT and LT) as the main-factor and harvesting time (6, 24, and 72 HAT) as sub-factor for each common waterhemp population. The percentage of herbicide absorbed and translocated were used to fit asymptotic regression, rectangular hyperbola (RHB), and linear model according to Kniss et al. using ‘drc’ and ‘qPCR’ packages in R. After fitting the data to these three models, the bias-corrected Akaike information criteria (AICc) of each model was obtained and compared. For analyzing both 2,4-D absorption and translocation, the RHB model was selected due to the lowest AICc values. The RHB model used is:

\[
\text{Absorption} = \frac{A_{\text{max}} \times t}{(10/90) \times A_{90} + t}
\]

\[
\text{Translocation} = \frac{T_{\text{max}} \times t}{(10/90) \times T_{90} + t}
\]

In the above equations, absorption is percent herbicide absorbed expressed in terms of percentage herbicide applied to the plant, A_{\text{max}} is the maximum herbicide absorption in time t, and A_{90} is the time required for 90% of the absorption to occur. Similarly, translocation is the percent herbicide translocated expressed in terms of percentage herbicide absorbed in the plant, T_{\text{max}} is the maximum herbicide translocation in time t, and T_{90} is the time required for 90% of the translocation to occur. A_{\text{max}}, A_{90}, T_{\text{max}}, and T_{90} parameters of WHR and WHS at each temperature regime were compared using the “compParm” function in the ‘drc’ package.

In metabolism experiments, chromatographs obtained from HPLC profiling were used for visual assessment of ^14C 2,4-D degradation. Percent parent ^14C 2,4-D present in each sample was determined and analyzed using GraphPad Prism 7.04® (GraphPad Software, San Diego, CA) at p = 0.05 and comparisons were made between HT and LT conditions in each biotype.

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Competing interests
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