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Investigation into interactions of environmental and application time effects on 2,4-D and dicamba-induced phytotoxicity and hydrogen peroxide formation

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Abstract

Application timing and environmental factors reportedly influence the efficacy of auxinic herbicides. In resistance-prone weed species such as Palmer amaranth (*Amaranthus palmeri* S. Watson), efficacy of auxinic herbicides recently adopted for use in resistant crops is of utmost importance to reduce selection pressure for herbicide-resistance traits. Growth chamber experiments were conducted comparing the interaction of different environmental effects with application time to determine the influence of these factors on visible phytotoxicity and hydrogen peroxide (H_2O_2) formation in *A. palmeri*. Temperature displayed a high degree of influence on 2,4-D and dicamba efficacy in general, with applications at the low-temperature treatment (31/20 C day/night) resulting in an increase in phytotoxicity compared with high-temperature treatments (41/30 C day/night). Application time across temperature treatments significantly affected 2,4-D–induced phytotoxicity, resulting in a ≥30% increase across rates with treatments at 4:00 PM compared with 8:00 AM. Temperature differential had a significant influence on dicamba efficacy based on visible phytotoxicity data, with a ≥46% increase with a high (37/20 C day/night) compared with a low differential (41/30 C day/night). Concentration of H_2O_2 in herbicide-treated plants was 34% higher under a high temperature differential compared with the low differential. Humidity treatments and application time interactions displayed undetected or inconsistent effects on visible phytotoxicity and H_2O_2 production. Overall, temperature-related influences seem to have the largest environmental effect on auxinic herbicides within conditions evaluated in this study. Leaf concentration of H_2O_2 appears to be generally correlated with phytotoxicity, providing a potentially useful tool in determining efficacy of auxinic herbicides in field settings.

Introduction

Maintaining optimal weed control with each herbicide application is a valuable strategy for agronomists not only to maximize cost-effectiveness by reducing the need for sequential applications, but also to manage herbicide resistance. Reduced herbicide efficacy has been directly linked to increased survival of weeds possessing potential resistance-conferring alleles, leading to exponential increases in the presence of weeds possessing these traits (Manalil et al. 2011; Neve and Powles 2005; Norsworthy et al. 2012). Many factors contribute to reduced herbicide efficacy, but among the most significant and relevant to current herbicide application patterns are environmental influences and diurnal variation in activity. In general, herbicides tend to be most effective under high-humidity and high-temperature regimes (Anderson et al. 1993; Johnson and Young 2002; Ritter and Coble 1981). However, these conditions are, by no means, uniform across seasons. Reduced herbicide efficacy has been reported with applications made at dawn and/or dusk (Dalazen and Merotto 2016; Johnston et al. 2018; Prasad et al. 1967; Sellers et al. 2003; Stewart et al. 2009). Unfortunately, dawn and dusk are times when cropping operations apply herbicides in order to cover large amounts of acreage in a timely manner and/or to reduce drift potential and decrease evaporation of spray droplets before herbicide can penetrate leaf tissues (Johnston et al. 2018). Upon the recent advent of auxinic herbicide–resistant crops, maintaining maximum activity will promote long-term utility of these

chemistries for agronomists (Bauerle et al. 2015; Johnston et al. 2018). However, more research is highly warranted on the interaction of different environmental and diurnal factors on auxinic herbicide efficacy. This is especially critical for weeds that cause major resistance problems.

Maintaining effective herbicide options for Palmer amaranth (*Amaranthus palmeri* S. Watson) control is one of the most challenging weed management problems in the southern United States. Control of *A. palmeri* was for a time achieved solely by using glyphosate after the introduction of glyphosate-resistant crops (Culpepper and York 1998; Ward et al. 2013). However, due to the evolution of glyphosate resistance in *A. palmeri* endowed by a novel gene-amplification mechanism (Gaines et al. 2010), control of this species with glyphosate is now nearly unachievable in most of the southern United States, warranting the adoption of other POST herbicide options. Resistance of *Amaranthus* species to several other herbicide mechanisms of action is also widespread throughout the United States, partly due to the high degree of genetic variability from obligate outcrossing and massive seed production (Assad et al. 2017; Culpepper et al. 2006; Foes et al. 1998; Giacomini et al. 2017; Heap 2014; Horak and Loughin 2000; Shoup et al. 2003; Webster and Grey 2015).

Increased genetic variability allows for an increased risk of selection for herbicide-resistant weed biotypes (Tranel and Wright 2002). It can be theorized that continued use of 2,4-D and dicamba in resistant crops will increase selection for herbicide-resistant alleles. Indeed, 2,4-D–resistant waterhemp [*Amaranthus tuberculatus* (Moq.) J. D. Sauer] has already been reported (Bernards et al. 2012; Figueiredo et al. 2017), so this phenomenon is no longer theoretical. Issues with *A. tuberculatus* resistance were preceded by the discovery of dicamba-resistant kochia [*Bassia scoparia* (L.) A. J. Scott] and wild mustard (*Sinapis arvensis* L.) (Cranston et al. 2001; Jasieniuk et al. 1995). Taking advantage of favorable environmental conditions and application timings when using herbicides would ensure maximum efficacy and would at least delay herbicide resistance. This is particularly important, because selection of *A. palmeri* with reduced susceptibility to auxinic herbicides has already been directly attributed to the use of sublethal rates of dicamba (Tehranchian et al. 2017).

One of the main phytotoxic effects associated with auxinic herbicide application is the formation of reactive oxygen species (ROS) (Song 2014). Increased activity of enzymes involved in ureide metabolism, fatty-acid oxidation, and jasmonic acid biosynthesis are generally considered a main source of ROS production (Pazmino et al. 2011; reviewed in Song 2014). One of the most prevalent herbicide-induced ROS species is hydrogen peroxide, production of which is triggered by abscisic acid–induced declines in photosynthesis and activity of nicotinamide adenine dinucleotide phosphate oxidases (Grossmann et al. 2001; Romero-Puertas et al. 2004). At present, there is a notable lack of research investigating the degree of ROS production in response to commercially applied auxinic herbicides. Investigation into the formation of hydrogen peroxide may yield important insights into the variability of 2,4-D and dicamba response across different environmental and diurnal regimes. The objectives of this research were to evaluate the degree of phytotoxicity and hydrogen peroxide formation from 2,4-D and dicamba treatments under different temperature, temperature differential, and humidity environments, as well as at different application timings.

Materials and Methods

Plant Material and Treatments

Seeds of a glyphosate-resistant *A. palmeri* population collected from Macon County, GA, were sown into 236-ml polystyrene cups containing commercial potting mix (Sun Gro® Professional Growing Mix, Sun Gro Horticulture, Agawam, MA) and thinned to 1 plant per cup following establishment. Plants were germinated and grown in a growth chamber set to day/night temperatures and humidity corresponding to environmental factors (see description of separate growth chamber conditions below) with light from 8:00 AM to 12:00 AM at 600 μmol m^{-2} s^{-1} throughout the experiment. Plants were fertilized once with a 20-20-20 fertilizer at a rate equivalent to 33.6 kg N ha^{-1}, and herbicide treatments were applied at the 3- to 5-leaf (~8 cm) stage.

Treatments were arranged in a randomized complete block design with four replications, with location in the growth chamber serving as the blocking factor. Herbicide treatments included dicamba or 2,4-D at 0.28 kg ai ha^{-1} or 0.56 kg ai ha^{-1}, with each herbicide/rate combination applied at either 8:00 AM, 8 h after initiation of darkness, or 4:00 PM, 8 h after initiation of light. All herbicide treatments contained a nonionic surfactant at 0.25% v/v. A nontreated control was included. Herbicide treatments were applied with a pipette to simulate a spray coverage of 280 L ha^{-1}. Spray coverage was calculated using the average leaf area of 4 plants (~52 cm^2). A total of seven 20-μl droplets were uniformly placed per plant to either side of the midrib on the adaxial side of each treated leaf. Herbicide treatments were applied inside the growth chamber to prevent any outside conditions (wind, differing temperature and humidity) from affecting herbicide penetration and absorption and to restrict observed effects to those originating from environmental factors, limiting any confounding outside effects on target plant physiology and metabolic processes. The application strategy was similar to that used by Parker et al. (2015) for absorption experiments. Herbicide treatments were made under separate growth chamber conditions to determine the effect of differing temperature, day/night temperature differential, and humidity on phytotoxicity and hydrogen peroxide formation. For high (41/30 C) and low (31/20 C) day/night temperature treatments, herbicides were applied at ambient humidity conditions (Supplementary Figures S1 and S2). For temperature differential treatments, herbicide applications were made under either 41/30 C (low, simulating summer) or 37/20 C (high, simulating spring) temperature differentials, at ambient humidity conditions for both temperature differential treatments. For determining the temperature differential effect, the previously collected high-temperature data were compared with data collected under the high temperature differential treatment. For humidity treatments, herbicide applications were made under humidity ranging from ~66% to 89% (high) or ~35% to 44% relative humidity (low), with a day/night temperature of 36/25 C. For the high-humidity treatment, a humidifier (Vicks® Warm Mist, Procter & Gamble, Cincinnati, OH) was placed in the growth chamber and filled with tap water twice a day. For the low-humidity treatment, a dehumidifier (DH-35K1SJE5, Hisense, Qingdao, China) was placed in the growth chamber with a drain hose connected to allow for constant moisture removal. Herbicide applications under each environmental factor were repeated twice in time.

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Phytotoxicity and Hydrogen Peroxide Determination

Visible phytotoxicity was determined at 3, 7, 10, and 14 d after treatment (DAT) using a scale of 0% to 100%, with 0% indicating no tissue damage and 100% indicating complete desiccation and plant death. At 14 DAT, plants were moved to the laboratory and harvested for determination of hydrogen peroxide concentration.

Hydrogen peroxide concentration *in planta* was determined using a colorimetric procedure adapted from Zhou et al. (2006). A total of 0.5 g of fresh leaf material from the most acropetal leaves was harvested and frozen in liquid nitrogen. Leaf material was ground into fresh powder with a precooled mortar and pestle to prevent thawing and then added to a 15-ml centrifuge tube along with 0.15 g of activated charcoal and 5 ml of a 5% trichloroacetic acid solution. Centrifuge tubes were then vortexed for 10 s before being centrifuged at 10,000 × *g* for 20 min at 4 C. Supernatant was then removed from centrifuge tubes and adjusted to pH 9 (determined to allow for maximum colorimetric resolution) using 30% ammonia solution. Adjusted supernatant was filtered using filter paper (Whatman No. 1, Whatman, Maidstone, UK) to remove any remaining solids. Filtrate was separated into two aliquots of 0.75 ml, and each was placed in a 2-ml centrifuge tube. One aliquot, treated with 6 µg of catalase dissolved in phosphate buffer to remove any hydrogen peroxide, was used as a blank. Catalase-treated aliquots were allowed to incubate at room temperature for 10 min. A colorimetric reagent was prepared containing 200 ppm 4-aminoantipyrine, 200 ppm phenol, and 100 ppm peroxidase (150 U mg^{-1}) dissolved in a 100 mM acetic acid buffer (pH 5.6). Following incubation of catalase-treated aliquots, 0.75 ml of colorimetric reagent was added to both aliquots. Aliquots were then capped and incubated in a water bath at 30 C for 10 min. Absorbance was read against the catalase blank at 505 nm and converted to "µM H_2O_2" using a linear standard curve prepared from H_2O_2 standards contained in the same solutions used for extraction from leaf tissue (Supplementary Figure S3). The standard curve was fit and analyzed using SigmaPlot (v. 11, Systat Software, San Jose, CA). Leaf weight was then used to finally convert to units of micromoles per gram of fresh weight (µmol g FW^{-1}).

Data Analysis

Visible phytotoxicity was subjected to analysis of covariance (ANCOVA) using JMP (JMP Pro v. 13, SAS Institute, Cary, NC), with DAT serving as the covariate. Separate overall analyses estimating the effect of the covariate and environmental factor, herbicide, herbicide rate, time of application, and their interactions were performed under each category of environmental factor (temperature, temperature differential, or humidity) at a significance level of 0.05. Separate ANCOVA analyses were then performed within each significant effect to test for covariate by treatment interactions in order to check homogeneity of slopes. In the case of significant covariate by treatment interactions, the null hypothesis (that the slope of the covariate within each significant treatment effect was statistically similar) was rejected, and the covariate by treatment interaction was retained in the model. If this significant interaction term was retained, slopes were separated using pairwise *t*-tests of indicator parameterization estimates. Overall means of each treatment are least-squares means, and means separation was carried out using pairwise *t*-tests at $\alpha = 0.05$.

For hydrogen peroxide data, ANOVA was carried out using the GLM procedure in JMP to determine significant treatment effects at a significance level of 0.05, with means separation carried out using pairwise *t*-tests at $\alpha = 0.05$. For both visible phytotoxicity data and hydrogen peroxide concentration, significant environmental factor by herbicide, herbicide rate, or application timing interaction resulted in a comparison of environmental factors within herbicide/rate combinations. In the case of insignificant environmental factor effects, only significant herbicide, herbicide rate, or application timing effect was subjected to pairwise *t*-tests. All graphs of visible phytotoxicity and hydrogen peroxide data were prepared using SigmaPlot.

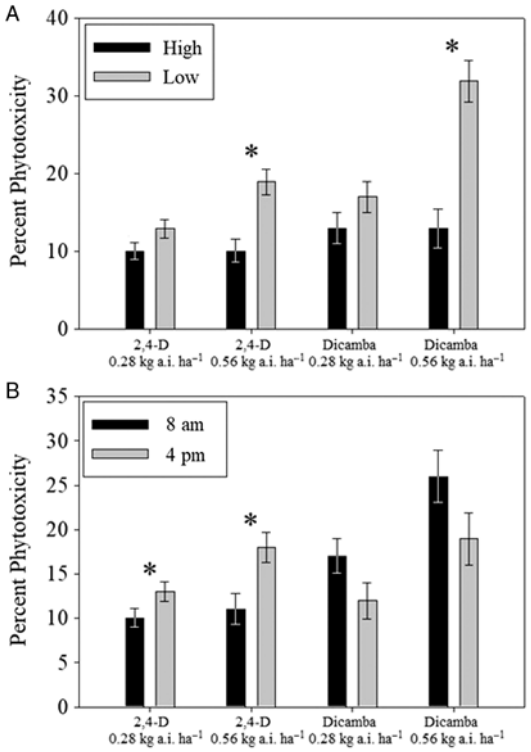

Figure 1. Means for effects of different temperature treatments pooled across application times (A) and application times pooled across temperature treatments (B) with different herbicide/rate combinations on visible phytotoxicity in *Amaranthus palmeri* in growth chamber experiments, 2018. Vertical bars represent standard error of the mean. Means are based on results from analysis of covariance analysis using days after treatment as a covariate. Asterisks represent significant differences within herbicide/rate combinations based on *t*-test results at $\alpha = 0.05$.

Results and Discussion

Temperature Effect

For visible phytotoxicity within temperature environments, no experimental run by temperature or experimental run by application time interactions were detected, thus data were combined over experimental runs. Significant temperature by herbicide and application time by herbicide interactions were detected in the overall analysis (Supplementary Table S1), thus means for temperature and application time are presented within each herbicide/rate combination. Temperature effects pooled across application times were only statistically significant within 2,4-D and dicamba treatments at the high rate (Figure 1). Phytotoxicity from 2,4-D application at 0.56 kg ha^{-1} was 19% and 10% for the low- and high-temperature treatments, respectively, while phytotoxicity from dicamba applications at 0.56 kg ha^{-1} resulted in 32% and 13% phytotoxicity for the low- and high-temperature treatments,

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Table 1. Slope comparisons for analysis of covariance results comparing effect of temperature treatment and application time within herbicide/rate combinations on percent phytotoxicity in *Amaranthus palmeri* in growth chamber experiments, 2018.

Effect				
Temperature	Herbicide	Temperature[a]	Slope[b]	Equation
	2,4-D 0.28 kg ha^{-1}	High	1.33 (0.25) B	$y = 1.33x - 1.19$
		Low	2.40 (0.30) A	$y = 2.40x - 7.37$
	P-value	Temperature	0.0627	
		DAT	<0.0001	
		Temperature*DAT	0.0069	
	2,4-D 0.56 kg ha^{-1}	High	1.39 (0.19) B	$y = 1.39x - 1.94$
		Low	3.40 (0.52) A	$y = 3.40x - 9.46$
	P-value	Temperature	<0.0001	
		DAT	<0.0001	
		Temperature*DAT	0.0006	
	Dicamba 0.28 kg ha^{-1}	High	1.97 (0.35) B	$y = 1.97x - 3.60$
		Low	3.50 (0.61) A	$y = 3.50x - 12.79$
	P-value	Temperature	0.1887	
		DAT	<0.0001	
		Temperature*DAT	0.0299	
	Dicamba 0.56 kg ha^{-1}	High	2.46 (0.45) B	$y = 2.46x - 7.32$
		Low	6.21 (0.77) A	$y = 6.21x - 20.16$
	P-value	Temperature	<0.0001	
		DAT	<0.0001	
		Temperature*DAT	<0.0001	
Application time	Herbicide	Application time[c]	Slope	Equation
	2,4-D 0.28 kg ha^{-1}	8:00 AM	1.45 (0.22) B	$y = 1.45x - 2.44$
		4:00 PM	2.28 (0.32) A	$y = 2.28x - 6.19$
	P-value	Time	0.0412	
		DAT	<0.0001	
		Time*DAT	0.0383	
	2,4-D 0.56 kg ha^{-1}	8:00 AM	2.43 (0.31)	$y = 2.43x - 5.93$
		4:00 PM		
	P-value	Time	0.0079	
		DAT	<0.0001	
		Time*DAT	0.0788	
	Dicamba 0.28 kg ha^{-1}	8:00 AM	2.73 (0.35)	$y = 2.73x - 8.25$
		4:00 PM		
	P-value	Time	0.0679	
		DAT	<0.0001	
		Time*DAT	0.0522	
	Dicamba 0.56 kg ha^{-1}	8:00 AM	4.28 (0.51)	$y = 4.28x - 13.65$
		4:00 PM		
	P-value	Time	0.0616	
		DAT	<0.0001	
		Time*DAT	0.2423	

[a]DAT, days after treatment. DAT serves as the covariate in analyses. Insignificant temperature or application time by covariate effects at a significance level of 0.05 resulted in one slope being reported for each temperature or application time effect within herbicide/rate combinations.
[b]Parentheses represent standard error of the slope estimate. Slope estimates followed by different letters differ statistically based on pairwise *t*-tests of indicator parameterization estimates at $\alpha = 0.05$.
[c]Time = application time.

respectively. Differences in application time pooled across temperature treatments were statistically significant within both rates of 2,4-D, with higher phytotoxicity resulting from 4:00 PM applications than from 8:00 AM applications (Figure 1). Phytotoxicity from 4:00 PM and 8:00 AM applications of 2,4-D at 0.28 kg ha^{-1} was 13% and 10%, respectively, while phytotoxicity from 4:00 PM and 8:00 AM applications at 0.56 kg ha^{-1} was 18% and 11%, respectively. There were no significant differences across application times for dicamba at either rate.

Because significant temperature level by herbicide and application time by herbicide interactions were detected in the overall analysis, separate ANCOVAs were performed on temperature level or application time within each herbicide treatment to determine differences in slopes (Table 1). Temperature by covariate interactions were detected for all herbicide/rate combinations, with higher slopes resulting from the low-temperature level in all cases. This indicates a faster progression of phytotoxicity at the low-temperature level compared with the high-temperature level. Application time by covariate interactions were only detected for 2,4-D at the 0.28 kg ha^{-1} rate, yielding a higher slope for the 4:00 PM application, indicating a faster progression of phytotoxicity compared with applications made at 8:00 AM.

For hydrogen peroxide concentration, significant experimental run by treatment interactions were not detected, thus data were combined over studies. Only herbicide effects were significant within the overall ANOVA (Supplementary Table S2). Dicamba application at the 0.56 kg ha^{-1} rate resulted in the highest H_2O_2 concentration at 1.62 µmol g FW^{-1}, followed by dicamba at the 0.28 kg ha^{-1} rate at 1.31 µmol g FW^{-1} (Figure 2). Dicamba applications at both rates resulted in significantly higher H_2O_2 concentration than in nontreated plants. In contrast, 2,4-D applications at 0.28 kg ha^{-1} and 0.56 kg ha^{-1} resulted in an H_2O_2 concentration of 0.97 µmol g FW^{-1}, which was statistically similar to the nontreated concentration (0.72 µmol g FW^{-1}).

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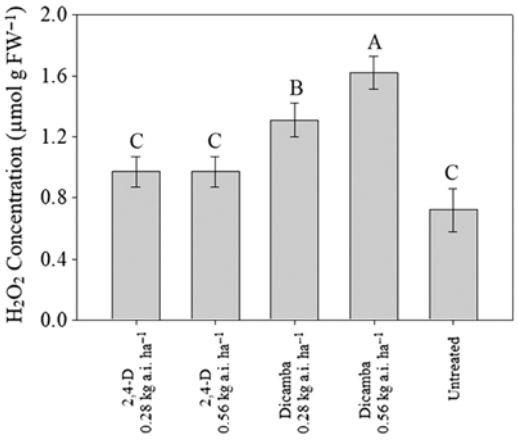

Figure 2. Means for hydrogen peroxide concentration in *Amaranthus palmeri* leaf tissue treated with 2,4-D and dicamba at two rates pooled across two temperature treatments and two application times in growth chamber experiments, 2018. Vertical bars represent standard error of the mean. The same letter above vertical bars indicates means do not differ statistically based on pairwise *t*-tests at $\alpha = 0.05$.

It appears that the rate effect with both 2,4-D and dicamba was more pronounced in the low-temperature treatment. Such trends may have to do with increased evaporation of herbicides at higher temperatures resulting in lesser rate effect. This is consistent with ANCOVA results, as higher slopes were observed for all herbicide treatments at the low temperature. This may be a result of increased spray droplet retention before evaporation compared with higher temperatures, resulting in faster realization of herbicidal activity. Indeed, previous research displayed a lesser degree of dicamba volatility at lower temperatures, although similar trends with 2,4-D have not been studied (Behrens and Lueschen 1979). Interestingly, improved translocation of 2,4-D, which may be correlated with increased phytotoxicity, was reported under high temperatures in hemp dogbane (*Apocynum cannabinum* L.) (Schultz and Burnside 1980). This is consistent with similar research that displayed improved translocation of triclopyr, picloram, and 2,4,5-T under higher temperatures (Radosevich and Bayer 1979). While we did not examine temperature in combination with translocation in this study, we observed no resulting increases in phytotoxicity with herbicides at the high temperatures that would support a positive correlation between temperature and translocation. Other past findings that conflict those presented this research include a reported increase in cotton (*Gossypium hirsutum* L.) and soybean [*Glycine max* (L.) Merr.] sensitivity to dicamba and 2,4-D in response to higher temperatures during exposure (Al-Khatib and Peterson 1999; Kittock and Arle 1977). As a result of conflicted findings across previous research, further study is necessary to determine mechanisms of temperature-induced differences in phytotoxicity.

Application time effects were observed only for 2,4-D and not dicamba, which could be attributed to the following: Dicamba, a more potent auxinic material in certain species, may have overcome any differences in auxinic herbicide efficacy across application timings due to the increased overall phytotoxicity associated with this herbicide (Leon et al. 2014; Nandula and Manthey 2002). Such an increase in dicamba activity may be associated with differential binding affinities to auxin-binding proteins (Webb and Hall 1995). This has important implications for growers, as applying auxinic herbicides with improved herbicidal activity in general (i.e., dicamba) may provide more flexibility in terms of application timing. A recommendation for increased rates of 2,4-D may be desirable if more potent auxinic materials are not labeled for certain situations. In such cases, crop oil adjuvants may reduce evaporation by improving herbicide penetration (Jansen et al. 1961). However, the mechanism responsible for reduced activity observed at times of lower temperature (i.e., 8:00 AM) demands further investigation. This likely is not due to reduced penetration of herbicide molecules as a result of any potentially increased evaporation. Differential translocation across application times has been reported and may describe such a phenomenon, although previous research has shown conflicting results on the nature of the correlation of phytotoxicity with translocation across times of application (Johnston et al. 2018).

A significant increase in H_2O_2 content was observed with the high rate of dicamba compared with the lower rate. It is somewhat expected that the H_2O_2 concentration would be increased with dicamba compared with 2,4-D, as dicamba is a more potent herbicide (Leon et al. 2014; Nandula and Manthey 2002). It is interesting that 2,4-D applications at either rate did not result in significantly higher concentration of H_2O_2 than in the nontreated; this may be due to either a reduced propensity for 2,4-D to trigger H_2O_2 formation or simply because rates of 2,4-D were not high enough to induce this phenomenon. It appears that across temperature regimes with temperature differentials held constant, dicamba is a more potent herbicide in *A. palmeri*, as shown by H_2O_2 data.

Temperature Differential Effect

For visible phytotoxicity under temperature differentials, no experimental run by differential or experimental run by application time interactions were detected, thus data were combined over studies. Significant temperature differential by herbicide and application time by herbicide effects were detected. As such, temperature differential and application time effects are presented within each herbicide/rate combination (Supplementary Table S1). Dicamba-induced phytotoxicity was significantly higher at both rates within the high temperature differential treatment, pooled across application times (Figure 3). With dicamba applications at 0.28 kg ha^{-1}, phytotoxicity was 13% and 19% for the low and high differentials, respectively, while at 0.56 kg ha^{-1} phytotoxicity was 13% and 25% for the low and high differentials, respectively. No significant differences in 2,4-D phytotoxicity were observed across temperature differential treatments. Significant differences in application time pooled across differential treatments were detected only with dicamba at the 0.28 kg ha^{-1} rate, with 21% phytotoxicity for 8:00 AM applications and 12% for 4:00 PM applications.

Because significant temperature differential by herbicide and application time by herbicide interactions were detected in the overall analysis, separate ANCOVAs were performed on temperature differential or application time within each herbicide treatment to determine slope differences (Table 2). Significant covariate by temperature differential interactions were detected for dicamba at both rates, and the slope was higher with the high differential at both rates. No significant covariate by temperature differential interactions were detected for 2,4-D at either rate. Covariate by application time interactions were only significant with dicamba at the 0.28 kg ha^{-1} rate, with a higher slope for 8:00 AM compared with 4:00 PM.

Only the temperature differential effect was significant in the H_2O_2 ANOVA. Concentration of H_2O_2 was significantly higher under the high temperature differential at 1.54 µmol g FW^{-1}

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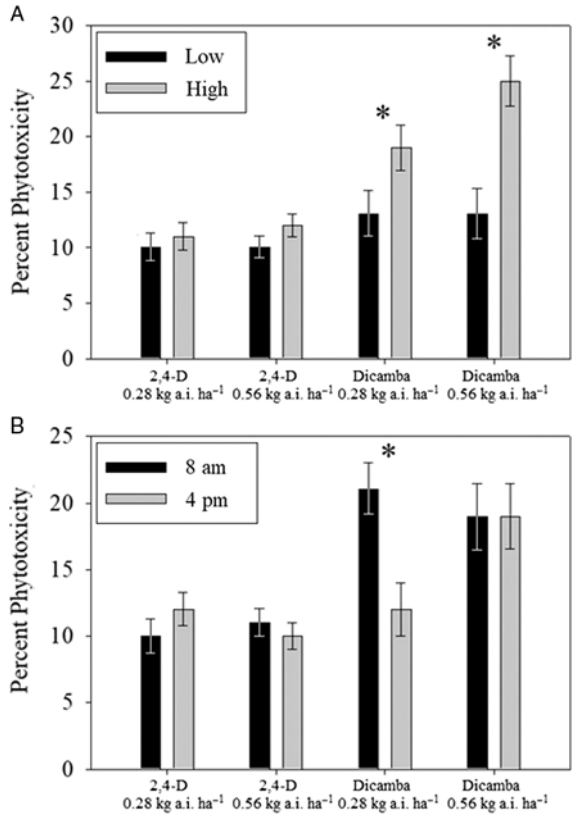

Figure 3. Means for effects of different temperature differential treatments pooled across application times (A) and application times pooled across temperature differential treatments (B) with different herbicide/rate combinations on visible phytotoxicity in *Amaranthus palmeri* in growth chamber experiments, 2018. Vertical bars represent standard error of the mean. Means are based on results from analysis of covariance analysis using days after treatment as a covariate. Asterisks represent significant differences within herbicide/rate combinations based on *t*-test results at $\alpha = 0.05$.

compared with the 1.15 μmol g FW^{-1} observed under the low temperature differential (Figure 4).

Little to no research has been published on the role of day/night temperature differential on efficacy of auxinic herbicides. Dicamba in general appears to be more sensitive to temperature differentials and application times across a temperature differential gradient, according to the data presented in this study. Increases in phytotoxicity of 46% and 92% were observed with dicamba at 0.28 kg ha^{-1} and 0.56 kg ha^{-1}, respectively, when the temperature differential was doubled (low to high treatment) (Figure 3). Significantly greater slopes under the high differential at both rates of dicamba are consistent, suggesting a faster progression of phytotoxicity compared with the low differential. It can be presumed that increasing the rate of dicamba would reduce the disparity in phytotoxicity between temperature differentials, although the effect of rate was nonexistent under the low temperature differential. On the contrary, a 32% increase in phytotoxicity was observed when the dicamba rate was increased from 0.28 kg ha^{-1} to 0.56 kg ha^{-1} under the high differential treatment.

Overall, these results suggest that dicamba is more rate responsive at high temperature differentials, in addition to being more potent in terms of phytotoxicity. Consistently, more H_2O_2 production was observed at the high temperature differential compared with the low temperature differential treatment when pooled across application times and herbicides. This further suggests that H_2O_2 production is well correlated with phytotoxicity. In terms of application time, dicamba provided higher control at 8:00 AM compared with 4:00 PM; however, this application time effect was removed upon increasing the rate. This is interesting, as the opposite trend was observed with 2,4-D across different temperature environments. The fact that the higher rate eliminated the application timing effect suggests that more active ingredient made it into the target site at 4:00 PM compared with the low rate. The results in this paper are consistent with previous research that displayed improved translocation, and subsequently phytotoxicity, of dicamba in *B. scoparia* under lower temperature regimes (Ou et al. 2018).

Humidity Effect

Experimental run by humidity treatment and experimental run by application time interactions were not detected for visible phytotoxicity data within humidity environments, thus results are combined over studies. Only herbicide effects were significant according to the overall ANOVA (Supplementary Table S1). Pooled across humidity treatments and application times, dicamba at the 0.56 kg ha^{-1} rate resulted in the highest phytotoxicity of 19%, with significantly reduced phytotoxicity from dicamba application at 0.28 kg ha^{-1} (11%) and 2,4-D application at 0.56 kg ha^{-1} (12%) (Figure 5). Application of 2,4-D at 0.28 kg ha^{-1} resulted in 9% phytotoxicity. Phytotoxicity from 2,4-D and dicamba applications at the low rate was statistically similar, as was phytotoxicity from 2,4-D at the high rate and dicamba at the low rate. Herbicide by covariate interactions were significant within the herbicide effect, with the highest slope resulting from dicamba application at 0.56 kg ha^{-1}, followed by a significant decrease with 2,4-D applications at 0.56 kg ha^{-1} (Table 3). Slopes of both dicamba at the low rate and 2,4-D at the high rate were statistically similar, as were slopes for dicamba and 2,4-D at the low rate.

Experimental run by humidity treatment interactions were significant ($P < 0.0001$) for H_2O_2 concentration, thus results are presented separately across studies. No significant effects were detected in Experiment 1; however, significant humidity by herbicide interactions were detected in Experiment 2 (Supplementary Table S2). As a result, humidity means are presented within herbicide/rate combinations for both studies for reference (Figure 6).

The lack of humidity effects suggests temperature may be the most influential environmental factor on differential control of *A. palmeri* with 2,4-D and dicamba. When herbicide effects were pooled across humidity treatments, trends followed as would be expected; increasing the rate of both 2,4-D and dicamba resulted in significant increases in phytotoxicity, with greater phytotoxicity resulting from dicamba in general. The lack of statistical significance in H_2O_2 production across treatments in Experiment 1 does not allow for any strong conclusions to be made on humidity effects. However, if H_2O_2 production is as well correlated with phytotoxicity as was observed for temperature-based environmental factors, there is likely a lack of true, reproducible significant differences across humidity levels using conditions employed in this research. The lack of humidity effects observed here is inconsistent with previous research on 2,4-D and dicamba that linked high humidity to decreased dicamba volatility and/or increased absorption, which would intuitively result in increased phytotoxicity (Al-Khatib et al. 1992; Behrens and Lueschen 1979; Pallas 1960). Low soil moisture often results in lower phytotoxicity from auxinic herbicides due to plant stress; however, whether or not this has an effect on plant perception of humidity and resulting effects

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Table 2. Slope comparisons for analysis of covariance results comparing effect of temperature differential treatment and application time within herbicide/rate combinations on percent phytotoxicity in growth chamber experiments, 2018.

Effect					
Temperature	Herbicide	Differential[a]	Slope[b]		Equation
	2,4-D 0.28 kg ha^{-1}	Low	1.63 (0.22)		$y = 1.63x - 3.17$
		High			
	P-value	Differential	0.4887		
		DAT	<0.0001		
		Differential*DAT	0.1833		
	2,4-D 0.56 kg ha^{-1}	Low	1.57 (0.18)		$y = 1.57x - 2.63$
		High			
	P-value	Differential	0.2297		
		DAT	<0.0001		
		Differential*DAT	0.3187		
	Dicamba 0.28 kg ha^{-1}	Low	1.97 (0.35)	B	$y = 1.97x - 3.60$
		High	3.71 (0.64)	A	$y = 3.71x - 11.98$
	P-value	Differential	0.0356		
		DAT	<0.0001		
		Differential*DAT	0.0198		
	Dicamba 0.56 kg ha^{-1}	Low	2.46 (0.45)	B	$y = 2.46x - 7.32$
		High	4.87 (0.66)	A	$y = 4.87x - 16.24$
	P-value	Differential	0.0006		
		DAT	<0.0001		
		Differential*DAT	0.0031		
Application time	Herbicide	Application time	Slope		Equation
	2,4-D 0.28 kg ha^{-1}	8:00 AM	1.63 (0.22)		$y = 1.63x - 3.18$
		4:00 PM			
	P-value	Time	0.2934		
		DAT	<0.0001		
		Time*DAT	0.267		
	2,4-D 0.56 kg ha^{-1}	8:00 AM	1.57 (0.18)		$y = 1.57x - 2.58$
		4:00 PM			
	P-value	Time	0.3518		
		DAT	<0.0001		
		Time*DAT	0.2037		
	Dicamba 0.28 kg ha^{-1}	8:00 AM	3.91 (0.65)	A	$y = 3.91x - 11.79$
		4:00 PM	1.88 (0.31)	B	$y = 1.88x - 4.30$
	P-value	Time	0.0011		
		DAT	<0.0001		
		Time*DAT	0.0054		
	Dicamba 0.56 kg ha^{-1}	8:00 AM	3.70 (0.43)		$y = 3.70x - 11.96$
		4:00 PM			
	P-value	Time	0.9275		
		DAT	<0.0001		
		Time*DAT	0.5544		

[a]Differential = temperature differential. DAT, days after treatment. DAT serves as the covariate in analyses. Insignificant temperature differential or application time by covariate effects at a significance level of 0.05 resulted in one slope being reported for each temperature differential or application time effect within herbicide/rate combinations.
[b]Parentheses represent standard error of the slope estimate. Slope estimates followed by different letters differ statistically based on pairwise *t*-tests of indicator parameterization estimates at $\alpha = 0.05$.

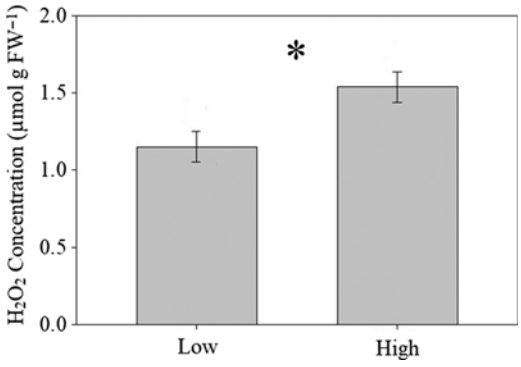

Figure 4. Means for hydrogen peroxide concentration in *Amaranthus palmeri* leaf tissue treated with 2,4-D and dicamba at two temperature differential treatments, pooled across rates and application times in growth chamber experiments, 2018. Vertical bars represent standard error of the mean. Asterisks represent significant differences within herbicide/rate combinations based on *t*-test results at $\alpha = 0.05$.

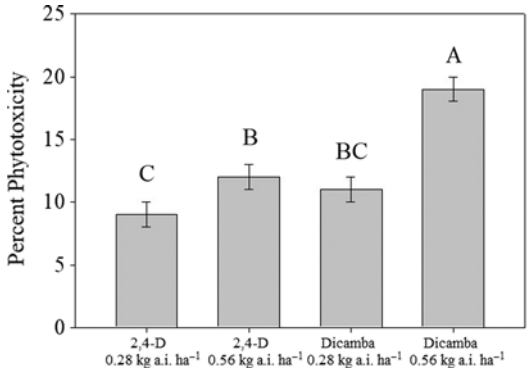

Figure 5. Means for effect of herbicide/rate combinations pooled across application times and two humidity treatments on visible phytotoxicity in *Amaranthus palmeri* in growth chamber experiments, 2018. Vertical bars represent standard error of the mean. Means are based on results from analysis of covariance analysis using days after treatment as a covariate. The same letter above vertical bars indicates means do not differ statistically based on pairwise *t*-tests at $\alpha = 0.05$.

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Table 3. Slope comparisons for analysis of covariance results comparing effect of herbicide/rate combinations pooled across humidity treatments and application times on percent phytotoxicity in growth chamber experiments, 2018.

Herbicide[a]	Slope[b]		Equation
2,4-D 0.28 kg ha^{-1}	1.46 (0.17)	C	$y = 1.46x - 3.60$
2,4-D 0.56 kg ha^{-1}	1.97 (0.24)	B	$y = 1.97x - 4.46$
Dicamba 0.28 kg ha^{-1}	1.92 (0.25)	BC	$y = 1.92x - 5.75$
Dicamba 0.56 kg ha^{-1}	3.62 (0.31)	A	$y = 3.62x - 12.02$
Herbicide	<0.0001		
DAT	<0.0001		
Herbicide*DAT	<0.0001		

[a]DAT, days after treatment. DAT serves as the covariate in analyses. Insignificant herbicide by covariate effects at a significance level of 0.05 resulted in one slope being reported for each herbicide/rate combination.
[b]Parentheses represent standard error of the slope estimate. Slope estimates followed by same letters do not differ statistically based on pairwise t-tests of indicator parameterization estimates at $\alpha = 0.05$.

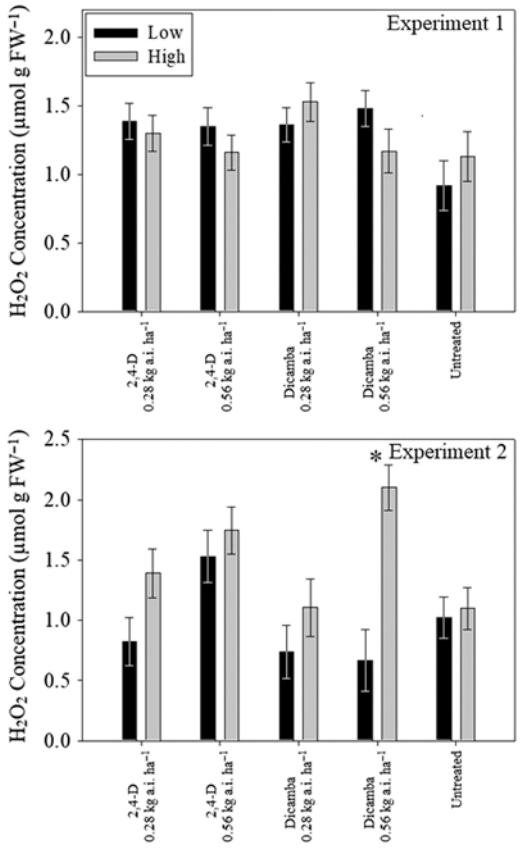

Figure 6. Means for hydrogen peroxide concentration in *Amaranthus palmeri* leaf tissue treated with 2,4-D and dicamba under two humidity treatments with different herbicide/rate combinations, pooled across application times in growth chamber experiments, 2018. Vertical bars represent standard error of the mean. Means are based on results from analysis of covariance analysis using days after treatment as a covariate. Asterisks represent significant differences within herbicide/rate combinations based on *t*-test results at $\alpha = 0.05$.

on auxinic herbicide activity demands further investigation (reviewed in Egan et al. 2014; Friesen and Dew 1966). The lack of findings in this study, in combination with the reports of previous research displaying increased soybean and cotton sensitivity to 2,4-D and dicamba under dry conditions, suggests this effect is relatively nuanced (Andersen et al. 2004; Auch and Arnold 1978; Kelley et al. 2005; Weidenhamer et al. 1989). Furthermore, moist conditions have been associated with increased sensitivity of cotton alone to 2,4-D and dicamba, convoluting trends even more (reviewed in Egan et al. 2014; Marple et al. 2007).

Overall, it appears that temperature-related effects provide the greatest environmental influence on factors tested in this research. In addition, application time effects are significant for 2,4-D and dicamba under different temperature and day/night temperature differential regimes, respectively. Increased rates of dicamba resulted in greater phytotoxicity to *A. palmeri* in general when negative application time effects were present; however, increasing rates appears to only improve phytotoxicity significantly at lower temperatures. As such, applying dicamba under lower temperatures and conditions where the day/night temperature differential (when possible) is greatest likely provides the best strategy for maximizing efficacy according to this research. Similarly, increasing 2,4-D rates appears to improve phytotoxicity more at low temperatures. In general, 2,4-D–induced phytotoxicity was insensitive to temperature differentials, indicating potentially greater flexibility for growers to apply this herbicide with greatly fluctuating day/night temperatures compared with dicamba. In addition, applications of 2,4-D appear to have greater efficacy at midday across temperatures. Further research is highly warranted on how these effects may differ with respect to other factors, including growth stage, presence of adjuvants, and other coinciding agronomic practices. Development of a rapid H_2O_2 assay in herbicide-treated plant tissues may provide a useful tool for agronomists to determine the efficacy of 2,4-D and dicamba applications. In the face of rapidly evolving herbicide resistance and the need for maintaining stewardship of auxinic herbicide chemistries, it is critical that agronomists maintain application strategies for 2,4-D and dicamba that take time of application and temperature influences into consideration.

Supplementary Material. To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2019.51.

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