Weed Science 2017 65:206–212

Recurrent Sublethal-Dose Selection for Reduced Susceptibility of Palmer Amaranth (*Amaranthus palmeri*) to Dicamba

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The management of glyphosate-resistant Palmer amaranth has been a challenge in southern United States cropping systems. Registration of dicamba-resistant crops will provide an alternative management option to control herbicide-resistant Palmer amaranth populations, particularly those having resistance to herbicide Groups 2, 3, 5, 9, 14, and 27. However, repeated use of sublethal doses of dicamba may lead to rapid evolution of herbicide resistance, especially in Palmer amaranth—a species with a strong tendency to evolve resistance. Therefore, selection experiments with dicamba were conducted on Palmer amaranth using sublethal doses. In the greenhouse, a known susceptible Palmer amaranth population was subjected to sublethal dicamba doses for three generations (P_1–P_3). Susceptibility of the individuals to dicamba was evaluated, and its susceptibility to 2,4-D was characterized. Based on the greenhouse study, following three generations of dicamba selection, the dose required to cause 50% mortality increased from 111 g ae ha^{-1} for parental individuals (P_0) to 309 g ae ha^{-1} for the P_3. Furthermore, reduced susceptibility of the P_3 to 2,4-D was also evident. This research presents the first evidence that recurrent use of sublethal dicamba doses can lead to reduced susceptibility of Palmer amaranth to dicamba as well as 2,4-D. Here, we show that selection from sublethal dicamba doses has an important role in rapid evolution of Palmer amaranth with reduced susceptibility to auxin-type herbicides.

Nomenclature: 2,4-D, dicamba, Palmer amaranth, *Amaranthus palmeri* S. Wats.
Key words: Low-dose selection, reduced susceptibility, whole-plant bioassay.

Palmer amaranth is the most troublesome and competitive weed of row crops in the southern United States (Klingaman and Oliver 1994) and has demonstrated the capacity to evolve resistance to several mechanisms of action (i.e., microtubule inhibitors, photosystem II inhibitors, acetolactate synthase inhibitors, 4-hydroxyphenylpyruvate dioxygenase inhibitors, 5-enolpyruvylshikimate-3-phosphate synthase inhibitors, and protoporphyrinogen oxidase inhibitors) (Heap 2016). Weed populations with multiple herbicide resistance to three or more mechanisms of action are increasingly common in the southern United States (Burgos et al. 2001; Norsworthy et al. 2008; Sosnoskie et al. 2011). New tools are needed for controlling Palmer amaranth and other herbicide-resistant dicotyledonous weeds in major crops. In light of multiple resistance evolution in Palmer amaranth, new technologies are in the process of commercialization, including soybean [*Glycine max* (L.) Merr.] and cotton (*Gossypium hirsutum* L.) cultivars resistant to soil-applied and over-the-top applications of the auxinic herbicide dicamba. Albeit, registration of dicamba for use in these crops is anticipated soon.

Auxinic herbicides (e.g. 2,4-D and dicamba) are structural analogues of the growth regulator indole-3-aceticacid (IAA) (Kirby 1980; Sterling and Hall 1997). Synthetic auxin-type herbicides selectively affect dicotyledonous plants by increasing endogenous auxin concentrations, leading to hormonal interactions in tissues (Grossmann 2010; Mithila et al. 2011). These herbicides at recommended field use rates cause rapid AUX/IAA repressor degradation and promote auxin-responsive gene expression (Chapman and Estelle 2009). Recent findings reveal that lethality of auxin-type herbicides on sensitive plants is due to unregulated auxin activity in addition to hyperaccumulation of plant hormones such

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DOI: 10.1017/wsc.2016.27

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as abscisic acid (Romero-Puertas et al. 2004). Leaf cupping, malformation, and stem epinasty are the typical symptoms of plants treated with auxin-type herbicides (Ahrens 1994). These herbicides also cause necrosis of terminal meristematic tissues followed by reduced root and shoot growth and, eventually, death of sensitive plants (Grabińska-Sota E et al. 2003). In addition to widespread use in burndown applications prior to crop planting, auxin herbicides have long been used to control many dicotyledonous weed species in grain crops such as wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), and grain sorghum [*Sorghum bicolor* (L.) Moench] (Mithila et al. 2011).

History has shown that repeated use of any single mechanism of action can quickly lead to resistance. Resistance can be endowed by a single or multiple genes (polygenic). Globally, there are biotypes of 25 dicotyledonous weed species that have evolved resistance to auxinic herbicides. Among them, only five weed species [cornflower, *Centaurea cyanus* L.; common lambsquarters, *Chenopodium album* L.; kochia, *Kochia scoparia* (L.) Schrad; prickly lettuce, *Lactuca serriola* L.; wild mustard, *Sinapis arvensis* L.] were reported to be resistant to dicamba (Heap 2016). Inheritance studies on dicamba-resistant weeds such as a wild mustard population from Manitoba, Canada (Jasieniuk et al. 1995), and a kochia population from Scotts Bluff County, Nebraska (Preston et al. 2009) suggested that resistance is due to alterations in a single gene locus when the herbicide is applied at the recommended field rate. Conversely, polygenic resistance may occur when recurrent sublethal doses select for the most tolerant plants within a population and when the selection agent is repeatedly employed over several generations (Busi et al. 2013; Neve and Powles 2005).

In addition to reducing application rates in an attempt to minimize herbicide costs, several other scenarios can lead to sublethal herbicide selection under field conditions, even when an herbicide has been applied at recommended rates. There are several factors, such as applications at larger than optimal weed size, applications under inappropriate weather conditions, and insufficient spray coverage, that may result in sublethal herbicide selection (Koger et al. 2004; Norsworthy et al. 1999, 2012).

Recurrent selection at sublethal herbicide doses, particularly in cross-pollinated species, accumulates several to many genes, some of minor effect, which collectively endow the resistant phenotype in survivors (Lande 1983; Macnair 1991; Neve et al. 2009; Taylor et al. 1989). This is in contrast to herbicide resistance endowed by a single or major gene (Powles and Yu 2010). Sublethal recurrent herbicide selection can result in resistance over three to four generations, as shown for glyphosate-resistance in Palmer amaranth and for acetyl-CoA carboxylase inhibitor resistance in rigid ryegrass (*Lolium rigidum* Gaudin) (Busi et al. 2013; Neve and Powles 2005; Norsworthy 2014). Recently, recurrent sublethal 2,4-D selection of cross-pollinated wild radish (*Raphanus raphanistrum* L.) for only three generations resulted in 2,4-D–resistant wild radish (Ashworth et al. 2016). With the anticipated use of dicamba in dicamba-resistant crops across vast acres targeting Palmer amaranth and other dicotyledonous weed species, experiments were conducted to: (1) assess the potential for sublethal dicamba doses to select for reduced susceptibility to the herbicide over multiple generations under laboratory and field conditions and (2) evaluate the selected population for reduced susceptibility to 2,4-D.

Materials and Methods

Parental Population. Seeds of a known dicamba-susceptible Palmer amaranth population were collected in September 2013 from a vegetable crop production field with no history of dicamba treatment at the Arkansas Agricultural Research and Extension Center, University of Arkansas, Fayetteville, AR (36°05'55.65'' N, 94°10'44.57'' W). Preliminary experiments, confirmed this population to be fully susceptible (100% mortality) to dicamba (Clarity®, BASF Ag Products, Research Triangle Park, NC) at 560 g ae ha^{-1} (the anticipated dicamba dose for dicamba-resistant crops) in a greenhouse study (unpublished data). This constituted the starting parental population (P_0). Seeds were germinated in plastic trays containing commercial potting mix (LC1, Sun Gro® Horticulture, AB, Canada) in a greenhouse at 35/25 C day/night temperatures and a 14 h photoperiod using high-pressure sodium lamps (400 $\mu\text{mol m}^{-2}\text{ s}^{-1}$). Seedlings at the 1- to 2-leaf stage were transplanted into 50-cell-plug plastic trays (54 by 28 by 6 cm) and maintained in the greenhouse. Plants were watered on a daily basis and fertilized once a week using a water-soluble fertilizer mix (Miracle-Gro® Products, Marysville, OH). Palmer amaranth plants in all experiments were treated with herbicide solutions at the 4- to 5-leaf stage. All herbicide treatments were applied using an automated research track sprayer with a boom mounted with two flat-fan 800067 nozzles (TeeJet Technologies) and calibrated to deliver 187 L ha^{-1} of herbicide solution at 270 kPa when moving at 1.6 km h^{-1}.

Generation of P_1–P_3. In the greenhouse, 1,152 P_0 seedlings were separated at the 4- to 5-leaf stage into three sets, with each set containing 384 seedlings. Each set of P_0 plants was treated with three doses of dicamba (70 [0.125X], 95 [0.17X], and 140 [0.25X] g ae ha^{-1}). Treated plants were maintained in the greenhouse for 21 days after treatment (DAT) under the same conditions described earlier. Dicamba at 140 g ae ha^{-1} resulted in highest plant mortality (47%) among the doses sprayed. The survivors of this dicamba dose were transplanted into larger plastic pots, grown to maturity, and cross-pollinated in a growth chamber (model CMP 6050, Conviron, Winnipeg, MB, Canada) to ensure pollination only among these plants. The growth chamber was programmed for a 14 h photoperiod with 900 μmol m^{-2} s^{-1} photon flux density at 35/25 C day/night temperatures. The seeds produced on these plants were termed P_1 seeds and were collected at maturity, air-dried at room temperature, and stored at 4 C for 2 wk to maximize germination. P_1 seeds served for the subsequent round of selection. Seedling establishment and dicamba treatment procedures were identical for all subsequent recurrent-selection processes. The P_1 seedlings were sprayed with three higher doses of dicamba, (i.e., 140 [0.25X], 280 [0.5X], and 560 [1X] g ae ha^{-1}), and survivors were selected from the 280 g ae ha^{-1} rate that resulted in 68% plant mortality. These survivors were grown to seed, constituting the P_2 generation, and a similar selection procedure at a higher dose was followed with the next generation to produce the P_3 generation (Table 1). As expected, the vast majority of the P_1, P_2, and P_3 were killed at the highest dicamba dose used in each of the three recurrent cycles of selection.

Table 1. Palmer amaranth survivors (%) selected under increasing dicamba doses 21 days after treatment.[a]

Selected populations	Dicamba dose (g ae ha^{-1})	Seedlings treated (no.)	Survivors (%)
P_0	140	384	53
P_1	280	300	32
P_2	420	380	71
P_3	560	280	21

[a] Selected plants were allowed to cross-pollinate and the seeds were used in subsequent cycle of selection.

Dicamba Dose–Response Studies for Low Dose–selected Populations. Dose–response studies were conducted to determine the response to dicamba of each of the P_0 to P_3 generations. The experiment was a randomized complete block design with 24 replications of individual plants and was conducted in two runs. Seedlings of each generation were transplanted into 24 cell plastic trays and treated at the 4- to 5-leaf stage with seven doses of dicamba. The herbicide doses were 35, 70, 140, 185, 280, 420, and 560 g ae ha^{-1}, which equates to 0.0625, 0.125, 0.25, 0.33, 0.5, 0.75, and 1X the anticipated field label rate of dicamba, respectively. All herbicide solutions contained a nonionic surfactant at 0.25% v/v (Induce®, Helena Chemical, Stuttgart, AR). Plant mortality was recorded 21 DAT.

Field Experiment. In a field experiment, soil naturally infested with glyphosate-resistant Palmer amaranth seed was collected in the autumn of 2011 from a 2 ha cotton field at the Northeast Research and Extension Center in Keiser, AR (35°40'30.73'' N, 90°04'48.92'' W). Soil was dried at ambient temperature and placed in cold storage at 4 C. Soil samples were taken from the soil surface to a 20 cm depth. The Palmer amaranth seed within these soil samples collected in 2011 served as the control seed. In each of the 2012 to 2015 years, this field was planted each spring with grain sorghum and treated with *S*-metolachlor (Dual II Magnum®, Syngenta Crop Protection, Greensboro, NC) at 1060 g ai ha^{-1} as a PRE herbicide immediately after planting to provide early-season control of Palmer amaranth while still allowing later cohorts to emerge. When most of the Palmer amaranth plants naturally infesting this field were approximately 45 cm in height, dicamba was applied to the entire field at 560 g ae ha^{-1}. As expected, the dicamba treatment caused high Palmer amaranth mortality each year (unpublished data), but some plants did survive and produced viable seed that fell to the soil surface in the normal manner before grain sorghum harvest. The same practices were followed in each of the three years (2012 to 2015); thus there were three consecutive years of dicamba treatment of Palmer amaranth in the field with efficacy and conditions that reflect normal practice.

Immediately following harvest of the third grain sorghum crop in October 2015, 10 soil samples were collected from this field and combined. The response to dicamba of Palmer amaranth seedlings originating from the 2011 vs. the 2015 soil samples were then compared over a range of dicamba doses under greenhouse conditions. The herbicide doses, application procedure, and greenhouse conditions were identical to the previously described recurrent low-dose dicamba-selection greenhouse experiments. The evaluation was conducted twice, and plant mortality were determined at 21 DAT.

Reduced Susceptibility to 2,4-D. Using procedures similar to those for dicamba dose–response studies, the P_0 seedlings and all three recurrent sublethal dicamba-selected populations (P_1–P_3) were grown in the greenhouse and treated with seven doses of the auxinic herbicide 2,4-D (Agristar® 2,4-D Amine 4, Albaugh, Ankeny, IA) at the 4- to 5-leaf stage. The 2,4-D doses were 70, 140, 280, 370, 560, 840, and 1120 g ae ha^{-1}, which equates to 0.0625, 0.125, 0.25, 0.33, 0.5, 0.75, and 1X the labeled field label rate of 2,4-D, respectively. Nonionic surfactant at 0.25% v/v was added to all spray solutions. Mortality data were recoded 21 DAT.

Statistical Analysis. Data of greenhouse and field experiments were subjected to ANOVA using PROC MIXED in SAS v. 9.1.3 (SAS, Institute, Cary, NC). Means were separated using Fisher's protected LSD at $\alpha = 0.05$. As a result of nonsignificant run by treatment interaction, data were pooled over two runs for each experiment. To determine the LD50 (dose required for 50% plant mortality) and LD90 (dose required for 90% plant mortality) of each population compared with P_0, mortality data were subjected to probit analysis using PROC PROBIT in SAS.

Results and Discussion

Recurrent Selection for Dicamba. Recurrent sublethal dicamba selection of Palmer amaranth for three generations resulted in individuals that survived dicamba at the anticipated labeled rate (Table 1). As expected, with the initial dicamba-susceptible Palmer amaranth plants (384 individuals termed the P_0 population), dicamba caused 47% mortality at the dose of 140 g ae ha^{-1}. The survivors (53%) were grown to maturity, cross-pollinated, and produced P_1 seed that served for the next generation of sublethal dicamba selection. After three rounds of this recurrent sublethal dicamba selection, the P_3 generation was compared with the P_0 population across a wide range of dicamba doses, and the P_3 generation was found to be less susceptible to dicamba than the P_0 population (Figure 1). Based on the LD50 values (Table 2), the P_3 population was more than 3-fold less susceptible to dicamba than the P_0 population. There were individuals of the P_3 population that survived dicamba doses well above that causing 100% mortality (420 [0.75X], and 560 [1X] g ae ha^{-1}) in the P_0 population. Based on the LD90 values, 213 and 838 g ae ha^{-1} of dicamba was required to kill 90% of P_0 and P_3 individuals, respectively (Table 2). The reduced susceptibility of Palmer amaranth to dicamba observed here is similar to the reduced susceptibility to glyphosate for this species following recurrent sublethal glyphosate selection (Norsworthy 2014).

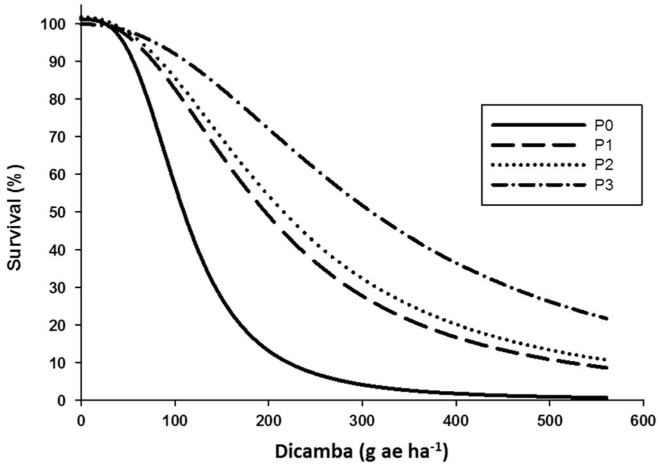

Figure 1. Dose–response curves for Palmer amaranth populations (P_0–P_3) selected following sublethal doses of dicamba in the greenhouse. Lines are the predicted values for percentage survival.

Table 2. Dicamba and 2,4-D doses required for 50% (LD50) and 90% (LD90) control of Palmer amaranth populations selected following sublethal doses of dicamba in the greenhouse.

Herbicide	Selected populations	LD50[a]	LD90[b]
		— g ae ha^{-1} —	
Dicamba	P_0	111 (92–130)[c]	213 (177–283)
	P_1	198 (165–235)	482 (378–724)
	P_2	221 (185–263)	546 (423–839)
	P_3	309 (257–382)	838 (610–1503)
2,4-D	P_0	241 (196–288)	569 (456–804)
	P_1	333 (278–391)	743 (599–1,057)
	P_2	416 (341–505)	1193 (892–1,961)
	P_3	489 (393–618)	1719 (1,183–3,396)

[a] LD50: dose of herbicide required to kill 50% of plants.
[b] LD90: dose of herbicide required to kill 90% of plants.
[c] Values in parenthesis indicate 95% confidence intervals.

Sublethal resistance selection occurs much more easily in species with obligate cross-pollination (Busi et al. 2013). Cross-pollination ensures resistance-endowing gene recombination in Palmer amaranth (Steckel 2007). Palmer amaranth male plants produce many pollen grains that can remain viable while traveling long distances from the paternal plant (Sosnoskie et al. 2007). Several studies characterizing the inheritance of glyphosate-resistance traits confirmed nuclear inheritance via pollen transmission in Palmer amaranth (Norsworthy 2014; Sosnoskie et al. 2012).

A nontreated control was not employed in the field selection at the same number of plants as those exposed to dicamba for each generation moved forward. It is acknowledged that lack of a nontreated control for each generation limits our ability to determine whether the frequency of tolerant individuals changed solely in response to the herbicide. Factors such as changes in effective population size and genetic drift may partially cause subtle changes in sensitivity to an herbicide over the course of several generations.

It is well established that low-dose recurrent herbicide selection leads to accumulation of "genes with small additive effects" in initially herbicide-susceptible cross-pollinated weed populations (Busi et al. 2016; Neve and Powles 2005; Orr and Coyne 1992). Evolution of herbicide resistance under low-dose selection scenarios is much slower in self-pollinated species such as wild oat (*Avena fatua* L.) than in cross-pollinated ryegrass (Busi et al. 2013, 2016). This is due to the negligible additive effect of gene traits in self-pollinated species. The characteristics of particular herbicides are also important in whether or not low-dose recurrent herbicide selection can lead to resistance (Yu and Powles 2014). Studies reveal rapid (three generations) recurrent low-dose resistance evolution for metabolizable herbicides such as diclofop or 2,4-D, likely because minor gene traits endowing a level of herbicide metabolism are present in susceptible plants and easily selected by low-dose recurrent selection (Busi and Powles 2009; Yu and Powles 2014). Dicamba is a metabolizable herbicide (Chang and Vanden Born 1971). This research demonstrates that overreliance on dicamba alone, especially at suboptimal doses, can select for reduced susceptibility in Palmer amaranth, as has recently been shown for 2,4-D in the cross-pollinated important weed wild radish (Ashworth et al. 2016). Ultimately, repeated use of any single weed control tactic, whether herbicide or otherwise, is not sustainable and must be integrated into a multifaceted approach (Norsworthy et al. 2012).

Recurrent Selection in the Field. The field experiment was designed to establish the effect of sublethal dicamba use over three consecutive generations. Even when an herbicide is applied at the recommended rate, certain field and environmental conditions can simulate sublethal dosage. A notable example is treating Palmer amaranth plants past the optimum growth stage for herbicide applications (>10 cm tall). Under typical field situations, Palmer amaranth is present at a range of sizes, and it is not uncommon to see a significant proportion of seedlings treated when they are large. Such conditions could eventually favor recurrent selection for reduced susceptibility over a number of generations. Results of the field experiment also corroborated the findings of the greenhouse experiment but suggested the likely influence of other factors under field conditions (see Supplementary Material).

Reduced Susceptibility to 2,4-D. Reduced dicamba susceptibility of the P_3 population compared with the P_0 population in the greenhouse was also evidenced as reduced susceptibility to 2,4-D (Figure 2). Based on the LD50 values, the P_3 was more than 2-fold less susceptible to 2,4-D compared with the P_0 parents (Table 2). At the labeled field rate (1120 g ae ha^{-1}), 2,4-D killed all P_0 plants, whereas 25% of the P_3 plants had survived the herbicide application as of 21 DAT. Cross-resistance of low dose–selected populations to herbicides with the same and different sites of action have been reported in

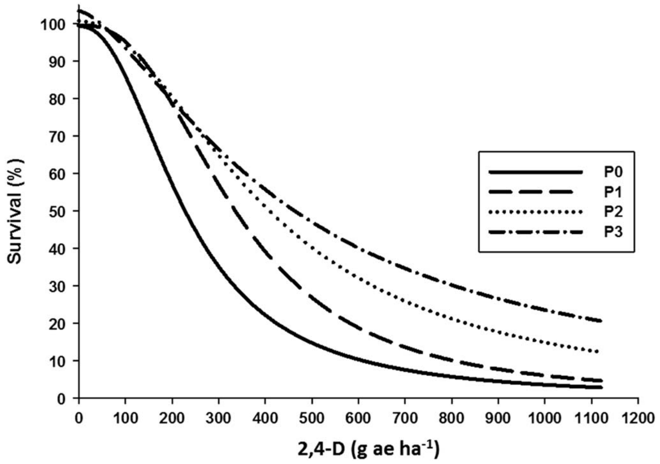

Figure 2. Dose–response curves using 2,4-D for Palmer amaranth populations (P_0–P_3) selected following sublethal doses of dicamba in the greenhouse. Lines are the predicted values for percentage survival.

rigid ryegrass, wild radish, and wild oat in Australia (Ashworth et al. 2016; Goggin et al. 2016; Neve and Powles 2005; Orr and Coyne 1992). Based on the dose–response results in this study, we can conclude that the endowed reduced susceptibility of P_3 at the anticipated rate of dicamba and the field rate of 2,4-D is likely a consequence of the same mechanism of resistance (this remains to be investigated).

In the majority of herbicide-resistance cases, inheritance of field-evolved resistance is single gene or a few dominant genes when herbicide is applied at the labeled field rate with high mortality (Preston and Mallory-Smith 2001). Therefore, mortality is achievable in susceptible plants except for rare individuals carrying strong resistance traits. However, use of herbicides at doses that are sublethal can lead to evolution of polygenic resistance. Agglomeration and expression of minor gene traits can collectively result in resistance and, in some cases, cross-resistance to similar and even dissimilar herbicide chemistries (Busi and Powles 2011; Busi et al. 2013; Norsworthy 2014; Preston et al. 1996). Here, we demonstrate the capability of a Palmer amaranth population to evolve reduced susceptibility to dicamba after three generations under a recurrent sublethal dicamba selection.

Similar to observations in other research (Gressel 2009; Neve and Powles 2005), these data strongly suggest that there will be evolutionary consequences if dicamba is not properly stewarded in dicamba-resistant crops. There is clearly the potential for rapid dicamba resistance evolution in Palmer amaranth if this herbicide is used at lower rates or applied in a manner that results in less than complete control, including when plants are at the improper growth stage. Cross-pollination will occur among the survivors. Non–target site herbicide resistance can evolve even in a small-sized weed population and can cause cross-resistance to other chemistries, particularly herbicides that can be metabolized (Gaines et al. 2014; Preston et al. 1996; Yu and Powles 2014). The genetic basis and associated mechanisms that led to dicamba resistance in the P_3 Palmer amaranth individuals are yet to be determined. However, it is well characterized that in dicotyledonous plants cytochrome P450 enzymes cannot metabolize auxin-type herbicides such as 2,4-D (Kelley and Riechers 2007; Kelley et al. 2004; Mithila et al. 2011). According to Subramanian et al. (1997), metabolism of dicamba by cytochrome P450s in monocot crops such as wheat and corn is negligible, and dicotyledonous crops are extremely sensitive to dicamba.

In conclusion, this is the first report that sublethal selection with dicamba results in reduced susceptibility in Palmer amaranth to this herbicide. There is also reduced susceptibility to 2,4-D. The U.S. Department of Agriculture has approved dicamba-resistant soybean and cotton cultivars for commercial production in the United States, and registration of dicamba for PRE and POST applications in these crops may occur soon. If dicamba is commercially approved for in-crop use, it is imperative that a stewardship program be developed and followed that protects dicamba and gives the best chance of longer-term sustainability of auxin-type herbicides. This study strongly discourages dicamba applications that provide less than complete Palmer amaranth control. The findings also emphasize the importance of integrating alternative herbicide mechanisms of action and nonherbicide tactics for Palmer amaranth control in dicamba- and 2,4-D-resistant crops.

Acknowledgments

Funding for this research was provided by Arkansas Soybean Promotion Board.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2016.27

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Received September 15, 2016, and approved November 10, 2016.

Associate Editor for this paper: Ramon G. Leon, University of Florida