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Comparative Analysis of 2,4-D Uptake, Translocation, and Metabolism in Non–AAD-1 Transformed and 2,4-D–Resistant Corn

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The Enlist™ traits provide 2,4-D resistance in several crops. Though corn is naturally tolerant to 2,4-D, the engineered trait conferred by the aryloxyalkanoate dioxygenase-1 (AAD-1) enzyme provides enhanced 2,4-D tolerance and confers resistance to the graminicide herbicide family, the aryloxyphenoxy-propionates. The objectives of this research were 2-fold: (1) measure and compare uptake, translocation, and metabolism of 2,4-D in Enlist™ (E, + AAD1) and non–AAD-1 transformed (NT, –AAD1) isogenic corn hybrids; and (2) and investigate the effect of glyphosate and/or the Enlist™ adjuvant system (ADJ) on these factors and corn injury. Uptake of radiolabeled 2,4-D acid applied alone in corn was not altered by the addition of ADJ when tank mixed at 24 h after application (HAA). By contrast, uptake of radiolabeled 2,4-D was significantly lower (69%) compared with 2,4-D plus ADJ (89%) at 24 HAA with a premixed formulation of 2,4-D choline plus glyphosate-dimethylamine (Enlist Duo™ herbicide [EDH]). Translocation of 2,4-D between the two corn hybrids was not different. E corn metabolized more 2,4-D (100% of absorbed) than NT corn (84%), and glyphosate did not alter 2,4-D metabolism. Furthermore, the metabolism of 2,4-D to nonphytotoxic dichlorophenol (DCP) and subsequent DCP-derived metabolites formed in E corn was examined. Injury to E corn is not typically observed in the field; however, injury symptoms were clearly evident in E corn (within 24 HAA) when formulated acetochlor was tank mixed with EDH, which correlated with an increase in 2,4-D uptake during this time period. In summary, the lack of injury in E corn following EDH applied alone may be attributed to a relatively low amount of 2,4-D uptake and the combination of natural and engineered 2,4-D metabolic pathways.

Nomenclature: 2,4-D; acetochlor; Enlist Duo™ herbicide; glyphosate; corn, *Zea mays* L.

Key words: Crop injury, detoxification, Enlist Weed Control System™, herbicide absorption, synthetic auxins

Enlist™ (E, +AAD1) corn contains the *aad-1* (*aryloxyalkanoate dioxygenase-1*) transgene, which encodes the bacterial AAD-1 enzyme that cleaves 2,4-D to the nonherbicidal metabolite, dichlorophenol (DCP) in vitro (Peterson et al. 2016; Wright et al. 2010). Enlist™ traits confer resistance to 2,4-D in several crops, including soybean [*Glycine max* (L.) Merr.] and cotton (*Gossypium hirsutum* L.), and provide enhanced tolerance in corn (Wright et al. 2010). Rapid metabolism of 2,4-D results in increased tolerance in E corn, therefore reducing the risk of typical auxin-like injury symptoms such as plant leaning, stalk brittleness, deformed brace roots, and reproductive malformations that may occur in non–AAD-1 transformed (NT, –AAD1) corn hybrids (Peterson et al. 2016). Importantly, the Enlist™ trait reduces growth-stage restrictions for POST applications in E corn (Wright et al. 2010). Unlike the AAD-12 enzyme used in transforming E soybean, the AAD-1 enzyme confers cross-resistance to the aryloxyphenoxypropionic acid (AOPP) subfamily of acetyl-CoA carboxylase inhibitors by selectively metabolizing their herbicidally active *R* isomers (Wright et al. 2010).

Injury from 2,4-D to E corn hybrids is not frequently observed in the field (Ruen et al. 2017). Field studies with E corn lines (containing initial transgene events) demonstrated tolerance to 3.4 kg ae ha^{-1} of 2,4-D per growing season (Wright et al. 2010). In contrast, E soybeans are occasionally injured following 2,4-D applications (Robinson et al. 2015), although yield is typically not affected. The injury occasionally observed in E soybean was not anticipated, because AAD-12 metabolizes 2,4-D more rapidly (~11-fold) than the AAD-1 enzyme in E corn hybrids (Wright et al. 2010). E corn demonstrated robust tolerance to single and sequential applications of formulated 2,4-D choline plus glyphosate-dimethylamine (Enlist Duo™ herbicide [EDH]) at 1X and 2X labeled rates (Ditmarsen et al. 2010), and yield of E corn was not affected by either single or sequential applications at 1X or 2X rates (Ruen et al. 2017).

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The primary difference between E and NT corn hybrids is the initial metabolic mechanism(s) in which 2,4-D is detoxified. NT corn can only metabolize 2,4-D initially through ring hydroxylation (Chkanikov et al. 1976; Feung et al. 1975; Montgomery et al. 1971; Roberts 1998; Schroder and Collins 2002) mediated by cytochrome P450 monooxygenases (Siminszky 2006), but E corn can also use the engineered bacterial AAD-1–mediated pathway that metabolizes 2,4-D to nonphytotoxic DCP (Wright et al. 2010; Zhou et al. 2016). As a result, the use of different metabolic pathways and enzymes will likely result in varying initial rates of metabolism by E corn compared with NT corn hybrids, but a direct comparison of 2,4-D metabolism in E vs. NT corn within a single study has not been reported in the literature.

The first objective of this research was to measure and compare the uptake, translocation, and metabolism of 2,4-D in E and NT corn hybrids. These biokinetic factors were measured in an isogenic corn hybrid lacking the AAD-1 trait in comparison with an E corn hybrid (+AAD1) to determine how AAD-1 may alter these physiological parameters. Uptake and translocation of 2,4-D in NT corn hybrids have been measured previously (Fang and Butts 1954; Hauser 1955; Robertson and Kirkwood 1970; Sargent and Blackman 1972) but not in direct comparison with an E corn hybrid. A second objective was to investigate the effect of glyphosate and/or the Enlist™ adjuvant system (ADJ) on these biokinetic factors and crop injury. The hypotheses underlying these objectives are: (1) a tank-mix combination or ADJ-increased 2,4-D uptake results in transiently elevated cellular concentrations of 2,4-D, which triggers foliar injury symptoms in corn if metabolic capacity is overwhelmed; (2) the presence of glyphosate in EDH does not affect the uptake, translocation, or metabolism of 2,4-D in E corn; (3) the initial rate and amount of 2,4-D metabolized in E corn is greater than in NT corn, which will subsequently affect whole-plant translocation of 2,4-D; and (4) the initial metabolic fate of 2,4-D is qualitatively different between E and NT corn due to the presence of the AAD-1 enzyme.

Materials and Methods

Whole-Plant Assay. E and NT corn hybrids (provided by Dow AgroSciences, Indianapolis, IN 46268) were grown to the V2 growth stage in a growth chamber under the following conditions: 16-h day/8-h night (28 C/26 C) and photosynthetically active radiation of 490 μmol m^{-2} s^{-1}. The E and NT hybrids used in this research are isogenic lines that share the same genetic background (including engineered glyphosate resistance), with the only difference being the pleiotropic 2,4-D- and AOPP-resistance traits conferred by *aad-1*. The V2 growth stage was selected to be consistent with previous research in corn and soybean (Skelton et al. 2013, 2014a). The first experiment focused on measuring 2,4-D uptake with various treatments containing 2,4-D, glyphosate, and ADJ, and the second experiment focused on a potential tank-mix combination that induced injury in E corn (Table 1).

Plants were treated with herbicides (Table 1) or ADJ (3% v/v when included) at V2 in a compressed-air research sprayer (DeVries Manufacturing, Hollandale, MN 56045) equipped with a TeeJet® 80015 EVS nozzle (TeeJet Technologies, Wheaton, IL 60187) calibrated to deliver 189 L ha^{-1} at 275 kPa. Plants were allowed to air-dry for 30 min prior to application of the radiolabeled herbicide treatments. Radiolabeled 2,4-D solutions (25.5 mM; specific activity 1.1 MBq $mmol^{-1}$) were the same as the whole-plant spray solutions (Table 1) but were spiked with uniformly ring-labeled (URL) [^{14}C]2,4-D (free acid). Herbicide solutions were applied using a glass syringe (Hamilton, Reno, NV 89502)

Table 1. Treatment list for whole-plant assays used to determine [^{14}C]2,4-D uptake and translocation in Enlist™ (E) and non–AAD-1 transformed (NT) corn hybrids.[a]

Experiment 1	Experiment 2
2,4-D choline only[b]	Enlist Duo™
2,4-D + ADJ[c]	Enlist Duo™ + Surpass NXT[f]
2,4-D + glyphosate[d]	
2,4-D + glyphosate + ADJ	
Formulated Enlist Duo™[e]	

[a] The herbicides listed refer to nonradiolabeled products only, but were spiked with [^{14}C]2,4-D as described in "Materials and Methods." Ammonium sulfate (AMS) (2.5% v/v, N-PAK AMS Liquid 34%; Dow AgroSciences, Indianapolis, IN 46268) was added to all treatments.

[b] 2,4-D choline, 1,065 g ae ha^{-1} (2,4-D choline [without formulation ADJ], 3.8 L; Dow AgroSciences, Indianapolis, IN 46268).

[c] Adjuvant (ADJ), 3% by volume (proprietary Enlist Duo™ ADJ Mixture; Dow Agrosciences, Indianapolis, IN 46268).

[d] Glyphosate, 1,120 g ae ha^{-1} (glyphosate-dimethylammonium salt [without formulation ADJ], 781.3 g L^{-1}; Dow AgroSciences, Indianapolis, IN 46268).

[e] Enlist Duo™ herbicide, 2,185 g ae ha^{-1} (Enlist Duo™, 400 g ae L^{-1}; Dow AgroSciences, Indianapolis, IN 46268).

[f] Surpass® NXT, 936 g ae ha^{-1} (Surpass® NXT, 839 g L^{-1}; Dow AgroSciences, Indianapolis, IN 46268).

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to deliver a total of 10 µl (100 Bq) divided into 33 droplets to the treated leaf. The second leaf was used as the treated leaf and was marked for clear identification during harvesting. Treatments without an adjuvant required that the treated leaf be fixed horizontally and applications delivered in a single 10 µl droplet (100 Bq) to prevent the solution from running off the leaf surface. This method maintained similar uptake levels when treatments with ADJ were used as a comparison (unpublished data).

After the radiolabeled solution was applied and allowed to air-dry, plants were returned to the growth chamber until sample collection at 1, 3, 6, 12, and 24 h after application (HAA). At sampling, the plant was divided into three parts: the treated leaf, plant material above the treated leaf portion ("above"), and plant material below the treated leaf ("below") portion. The treated leaf consisted of the entire leaf surface (from the collar to the leaf tip) to which the radiolabeled herbicide solution had been applied. The "above" and "below" portions were determined by dividing the remaining plant tissues into two fractions based on the collar of the treated leaf. The treated leaf was thoroughly rinsed immediately with 3 ml of a water:methanol solution (80:20 v/v) for 45 s in a 25-ml glass scintillation vial to remove any remaining [^{14}C]2,4-D material from the leaf surface, and excess water:methanol solution on the leaf surface was also collected in the scintillation vial. The aqueous methanol rinse solution was then analyzed via liquid scintillation spectrometry (LSS; Model 1900 TR, Packard Instrument, Meriden, CT 06450) to determine the amount of [^{14}C]2,4-D remaining on the surface. The treated leaf, above, and below portions were air-dried before combustion for 4 min in a biological oxidizer (Model OX-500, R.J. Harvey Instrument, Hillsdale, NJ 07462) and analysis via LSS to quantify the amount of ^{14}C-labeled material within each plant portion. Total radioactive material recovery, which was determined by summing the amount of ^{14}C-labeled material recovered from each step, averaged 90% for all experiments.

In a separate study, additional E and NT corn plants were analyzed with photostimulated luminescence (PSL) to qualitatively determine the location of ^{14}C-labeled material within the plant at 24 HAA. E and NT plants were treated as in the whole-plant assays, but a higher amount of [^{14}C]2,4-D was used (416 Bq leaf^{-1}) to facilitate imaging. After being air-dried, samples were placed on a phosphorimage exposure cassette (Molecular Dynamics) for 48 h before PSL analysis (Typhoon 9400 Variable Mode Imager, Amersham Biosciences, Buckinghamshire, UK). Following PSL analysis, the dried plant samples were oxidized to quantify the amount of ^{14}C material (as percent absorbed) in each portion as an additional reference to the qualitative phosphorimage results.

Excised-Leaf Assays

Metabolism of 2,4-D in E and NT Corn. Due to the difference in 2,4-D uptake among treatments recorded in the preliminary whole-plant assay, an excised-leaf assay was developed to normalize the amount of [^{14}C]2,4-D within the plant tissue examined and produce more accurate metabolism-degradation results. The method used for the excised-leaf assay was similar to ones previously described for corn (Kreuz and Fonne-Pfister 1992) as well as with a dicot weed, tall waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] (Ma et al. 2013, 2015). Both hybrids were grown in the growth chamber to the V2 growth stage to maintain consistency with the whole-plant assay earlier in this paper, and the second leaf was used to measure 2,4-D metabolism. The leaf to be treated was cut from the stem at the collar, then placed under water and cut again to ensure that air was not trapped in the vascular tissue (Kreuz and Fonne-Pfister 1992). The excised leaf assay was divided into three treatment stages, each occurring in the growth chamber: (1) 0.1 M Tris-Cl buffer (pH 6.5) preincubation, (2) ^{14}C-herbicide "pulse" solution, and (3) distilled water (allowing metabolism to occur) as described in the following paragraph.

After the leaf to be treated was removed from the plant, it was inserted into a 1.5-ml plastic tube containing 200 µl of the 0.1 M Tris-Cl buffer (pH 6.5) for 0.5 h to acclimate to the solution and pH. The leaf was then transferred to a new 1.5-ml tube containing 100 µl (4.2 kBq) of the ^{14}C-herbicide solution for 1 h. The herbicide "pulse" solution consisted of 25 µM [^{14}C]2,4-D acid (specific activity 1.4 GBq/mmol) in 0.1 M Tris-Cl buffer (pH 6.5). Herbicide treatments were either [^{14}C]2,4-D acid only or [^{14}C]2,4-D acid plus nonlabeled glyphosate acid (35.5 mM). After 1 h, virtually all the herbicide solution had been absorbed, and the remaining amount was quantified by LSS to determine uptake. Finally, after 1 h the leaves were either washed immediately with distilled water and frozen in liquid nitrogen (for the 1-h samples) or transferred to a new tube containing 1.5 ml of distilled water and incubated in the growth chamber for either 2, 5, 11,

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or 23 h until sample collection (1, 3, 6, 12, and 24 HAA total incubation times). Distilled water was added throughout this stage to ensure that the leaf had a constant supply of water and was not dehydrated. Distilled water was used instead of half-strength Murashige and Skoog (MS) salt solution, as described previously for tall waterhemp (Ma et al. 2013, 2015), because injury symptoms developed when the MS salt solution was used with this assay in our experimental conditions.

At each sampling time, the leaf was removed from the solution and processed to extract ^{14}C-labeled material. The leaf was frozen with liquid nitrogen and homogenized with a glass rod, and radioactivity in the leaf tissue was extracted with 14 ml of an acetone:water solution (90:10 v/v) for 16 h at –4°C. Following this extraction, samples were centrifuged at 12,000 × *g* for 10 min, resulting in two distinct phases: a solid pellet (nonextractable) and liquid (extractable) portion. The nonextractable portion was allowed to air-dry before oxidization to determine the amount of ^{14}C material remaining as bound residues. The liquid portion was analyzed using reverse-phase high-pressure liquid chromatography (RP-HPLC) to determine [^{14}C]2,4-D metabolism. Samples for RP-HPLC analysis were generated by concentration at 40 C with a rotary evaporator (Rotavapor R-200, BÜCHI, Flawil, Switzerland) until a final volume of 0.5 ml was reached. Acetonitrile:water (50:50 v/v) was added to adjust the final volume of the extracts to 1.25 ml, and extracts were centrifuged at 10,000 × *g* for 10 min. Total radioactivity in each sample was measured by LSS, and each sample was normalized to 38.5 Bq to inject equivalent amounts of radioactivity (in a final volume of 10 µl) onto the column during each run. Final recovery of ^{14}C material ranged from 92% to 98%.

RP-HPLC was performed on a Perkin-Elmer Flexar LC (Model N2910401, Perkin Elmer, Akron, OH 44311) with an Alltima™ C18 column (4.6 by 150 mm, 5 µm; Alltech, Columbia, MD 21044) at a flow rate of 1 ml min^{-1}. Eluent A was 0.1% (v/v) formic acid in water and eluent B was acetonitrile. The elution profile was as follows: step 1, 80% A:20% B (v/v) for 12 min; step 2, 60% A:40% B for 5 min; step 3, 30% A:70% B for 2 min; step 4, 10% A:90% B for 3 min; step 5, 80% A:20% B for 2 min (24 min total). Radiolabeled compounds were detected with a β-RAM Radio-HPLC Detector (Model 4, LabLogic, Brandon, FL 33511) and Ultima-Flo M cocktail (Perkin-Elmer, Waltham, MA 02451). [URL][^{14}C]2,4-D acid displayed a retention time of 21 min under these conditions. Figure 1 shows a typical RP-HPLC chromatogram of 2,4-D metabolism in E and NT corn hybrids at 1 and 24 HAA.

Metabolism of DCP in E and NT Corn. The excised-leaf assay was also used to determine the metabolism of DCP. DCP is the first metabolite formed in E corn by the AAD-1 enzyme (Wright et al. 2010; Zhou et al. 2016) and would indicate whether DCP is metabolized in a similar fashion between corn hybrids regardless of the transgene, as determined previously in 2,4-D–resistant and 2,4-D–sensitive cotton (Laurent et al. 2006). The assay used 25 µM [^{14}C]DCP (specific activity 1.2 GBq mmol^{-1}) to measure metabolism at 1 and 24 HAA. Samples generated from excised-leaf assays were analyzed by HPLC as previously described to separate radiolabeled metabolite(s) from parent 2,4-D in E and NT corn but with modifications to provide greater resolution, including a different column (Hypersil Gold™ C4, 4.6 by 250 mm, 5 µm; Thermo-Fisher Scientific, Waltham, MA 02451) and mobile-phase gradient. The modified elution profile was as follows: step 1, 85% A:15% B (v/v) for 9 min; step 2, 70% A:30% B for 10 min; step 3, 40% A:60% B for 10 min; step 4, 5% A:95% B for 5 min; step 5, 98% A:2% B for 1 min (35 min total). [URL][^{14}C]2,4-D displayed a retention time of 15 min and [URL][^{14}C]DCP displayed a retention time of 13.5 min under these conditions.

Statistical Methods. Treatments were arranged in a completely randomized design, and data from each independent experiment were combined and analyzed. All experiments were conducted twice and contained three replications for each treatment combination. Uptake (U) and metabolism (M) biokinetic data were analyzed using nonlinear regression methods using the Michaelis-Menten model (inverse hyperbolic curve; Equation 1) (Kniss et al. 2011; Ritz et al. 2015) using the 'drc' package of R (R Development Core Team 2014; Ritz and Streibig 2012).

$$Y = (\alpha \times t)/(\beta + t) \quad [1]$$

In the model (Equation 1), Y represents the percentage of 2,4-D uptake or metabolism at time t, α is the parameter that estimates the maximum amount of Y, and β is the parameter that estimates the t to reach 50% α. In this model, α corresponds with the U_{max} and M_{max} values, which describe the maximum amount of 2,4-D uptake or metabolism,

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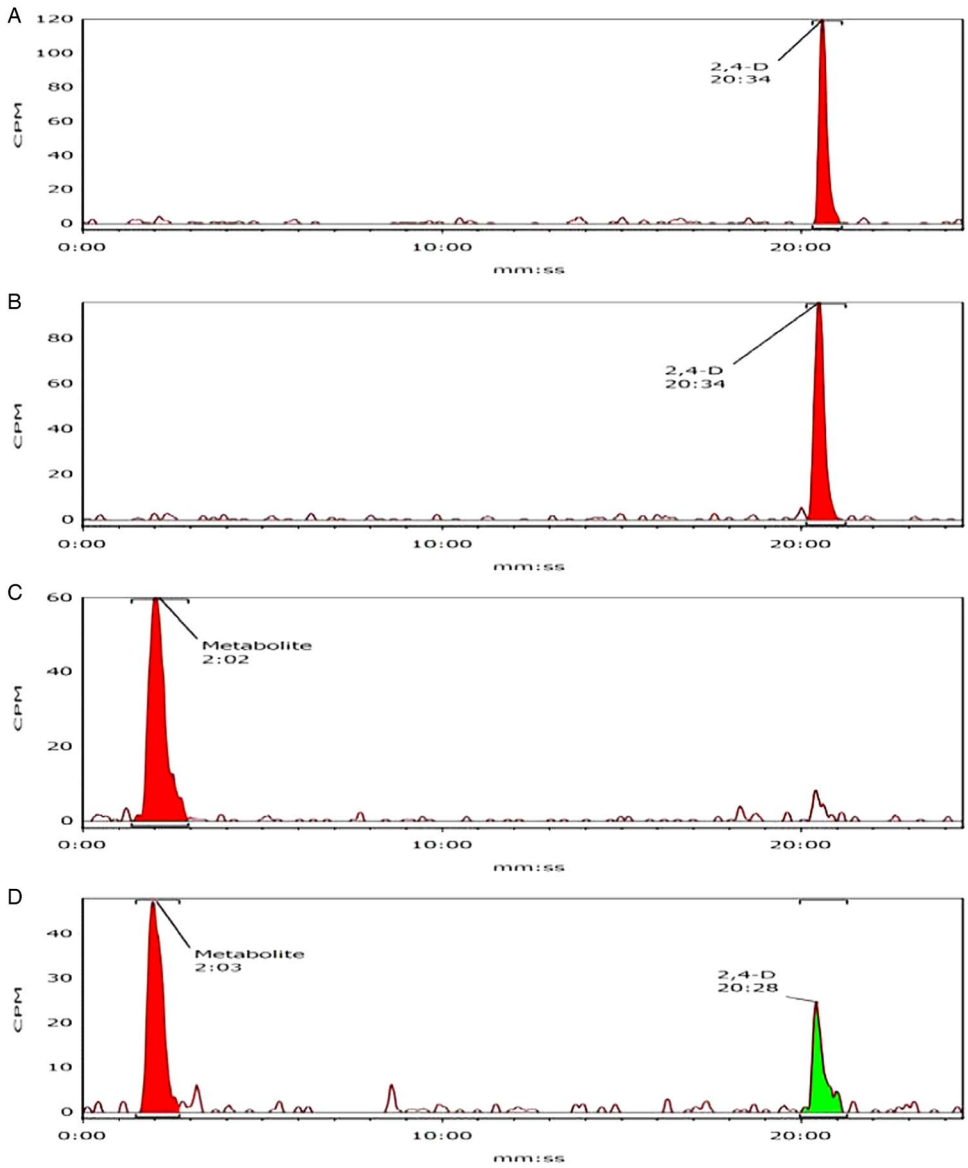

Figure 1. Representative reverse-phase high-pressure liquid chromatography radiochromatograms from the excised-leaf assay to determine parent [^{14}C]2,4-D (retention time 21 min) remaining at each time point. (A) Enlist™ (E) corn at 1 HAA, (B) non–AAD-1 transformed (NT) corn at 1 HAA, (C) E corn at 24 HAA, and (D) NT corn at 24 HAA. Note that the "Metabolite" peak eluting at approximately 2 min in C and D likely contains more than one radiolabeled compound. Abbreviation: CPM, counts per minute.

and β corresponds with U_{50} and M_{50} values, which describe the initial rate of 2,4-D uptake or metabolism. Parameters of different models were compared using pairwise differences ($\alpha = 0.05$) with the *compParm* function in R. Data for translocation and nonextractable ^{14}C material were analyzed with SAS (SAS v. 9.4, SAS Institute, Cary, NC 27513), using PROC MIXED in a split-plot design with the whole plot being each time point and the subplot being the treatment combinations. Means were separated using Tukey's honest significant difference test at $\alpha = 0.05$. Experiment by treatment interactions were not detected, and results were pooled for both experimental replications.

Results and Discussion

Whole-Plant Assays

Uptake and Translocation of 2,4-D in E and NT Corn. The amount (U_{max}; $P = 0.25$) and rate (U_{50}; $P = 0.43$) of 2,4-D uptake measured across all treatments were not different between E and NT corn hybrids. In contrast, U_{max} ($P = 0.02$) but not U_{50} ($P = 0.19$) differed among treatments (Table 2). The only significant difference in U_{max} occurred between the 2,4-D plus ADJ and EDH treatments, while treatments without the ADJ had similar uptake levels as treatments with the ADJ (Table 2). At 1 HAA, all treatments displayed equivalent 2,4-D

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Table 2. Nonlinear regression parameters for 2,4-D uptake from whole-plant assays with Enlist™ (E) and non–AAD-1 transformed (NT) corn hybrids.

Treatment	U_{max}[a,b]	U_{50}[c,d]
2,4-D only	86.5 ef	3.4
2,4-D + ADJ	101.2 e	2.5
2,4-D + glyphosate	90.4 ef	3.9
2,4-D + glyphosate + ADJ	87.6 ef	2.6
Formulated Enlist Duo™	75.6 f	5.1

[a] Parameter corresponding to the modeled maximum 2,4-D uptake (% recovery) of treatment.
[b] Values followed by the same letter within each experiment are not significantly different as determined by Fisher's LSD test ($\alpha = 0.05$).
[c] Parameter corresponding to time (h) for treatment to reach 50% U_{max}.
[d] U_{50} values for treatments were not statistically different ($P = 0.19$).

uptake levels, but from 3 to 12 HAA, the EDH treatment had significantly lower uptake levels than any other treatment (Figure 2). By 24 HAA, the EDH treatment exhibited significantly lower 2,4-D uptake (69%) than the 2,4-D plus ADJ treatment (89%; Figure 2). Thus, the significant increase in radiolabeled 2,4-D uptake derived from the ADJ alone previously measured in soybean (about 36%; Skelton et al. 2013, 2014a) was not detected in corn.

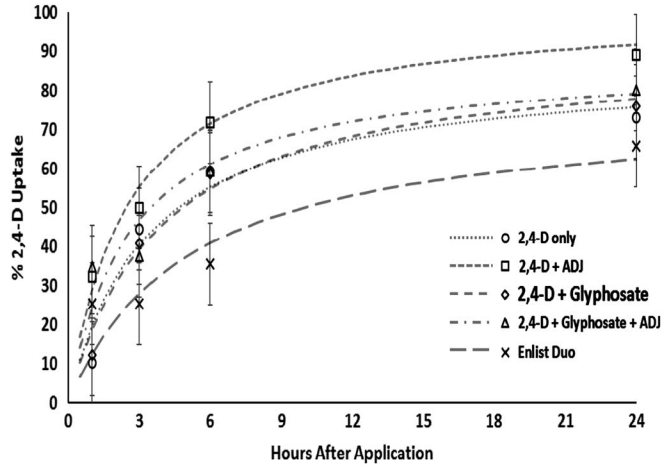

Figure 2. Uptake of 2,4-D among the five treatments from the whole-plant assay with Enlist™ (E) and non–AAD-1 transformed (NT) corn during the 24-h time course. Symbols represent the average uptake at each time point among treatments (ADJ, adjuvant from Enlist Duo™). All treatments were applied at the same rate of 2,4-D choline (1,065 g ae ha^{-1}), glyphosate (1,120 g ae ha^{-1}), or ADJ (3% v/v); AMS (2.5% v/v) was included in each treatment. Treatment means are the result of combining data from two separate experiments with three replications per individual treatment. Vertical bars represent the standard error of the mean.

The EDH treatment resulted in the least amount (lowest U_{max}) of 2,4-D uptake in corn leaves among treatments examined (Table 2), which is in contrast to previous measurements in soybean (Skelton 2013, 2014a). The lack of an increase in 2,4-D uptake in corn from ADJ may be related to several factors: (1) the ADJ targeted dicot weed control instead of grass control (Devkota and Johnson 2016), since 2,4-D POST does not control grasses; and/or (2) development and optimization of the ADJ was aimed at improving foliar glyphosate uptake, which was not measured in this study or previous studies (Skelton 2013, 2014a). A lack in adjuvant-enhanced uptake has been measured in other plants with varying combinations of herbicide–adjuvant physiochemical properties (Wang and Liu 2007), but additional mechanistic research is required to better understand this anomaly.

Translocation of ^{14}C material was not significantly different ($P = 0.27$) between E and NT corn. The amount of ^{14}C-labeled material remaining in the treated leaf was equivalent for both hybrids (Table 3). However, a significant difference ($P \leq 0.0001$) was determined between above and below portions in the limited amount of radiolabeled material translocated from the treated leaf (averaged for both E and NT corn). For example, the amount of radiolabeled material was equally distributed between the two parts at 1 and 3 HAA, but the amount in the above portion was greater than the below portion at 6 and 24 HAA. Figure 3 illustrates a phosphorimage analysis of E and NT corn plants treated with [^{14}C]2,4-D at 24 HAA (different study than Table 3). The location of the ^{14}C material was heavily concentrated in the treated leaves (Figure 3A and B; 83% and 94%), but a

Table 3. Distribution of ^{14}C material from whole-plant assays using Enlist™ (E) and non–AAD-1 transformed (NT) corn hybrids.

		Hours after application			
Hybrid	Portion	1	3	6	24
		% ^{14}C material in plant[a,b]			
NT	Treated leaf	97.3	100	96.0	92.6
	Above	0.7	0.4	1.8	3.8
	Below	0.4	0.3	0.6	2.3
E	Treated leaf	99.3	100	95.9	95.0
	Above	1.3	1.0	1.6	3.8
	Below	0.2	0.1	0.4	1.5

[a] Significant difference not detected between corn hybrids in amount of ^{14}C material remaining in the treated leaf ($P = 0.27$).
[b] Significant interactions were not detected between hybrid, plant portion, and hours ($P = 0.75$).

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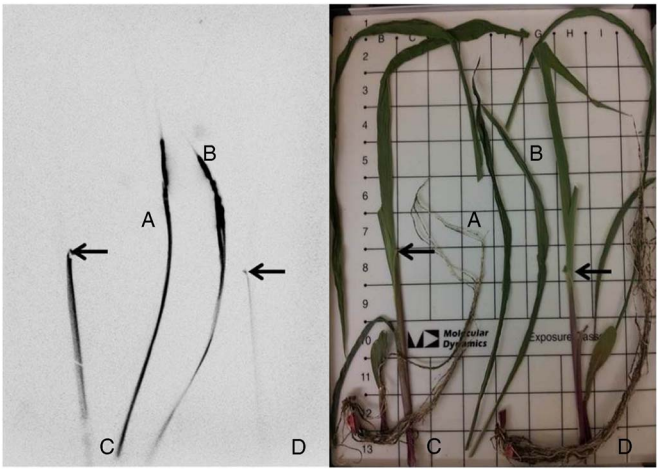

Figure 3. Phosphorimages and pictures of Enlist™ (E) and non–AAD-1 transformed (NT) corn seedlings at 24 HAA treated with [^{14}C]2,4-D (different study than data shown in Table 3). Areas of increased intensity indicate a relatively higher amount of radiolabeled material. (A) NT corn, treated leaf; (B) E corn, treated leaf; (C) NT corn, above and below treated leaf portion; and (D) E corn, above and below treated leaf portion. Both E and NT roots were analyzed but were not visible on the phosphorimage due to the low amounts of ^{14}C material (below the detection threshold). Black arrows indicate the point of attachment for the treated leaves. Plant portions were oxidized to determine the percent of ^{14}C material of total ^{14}C material absorbed and are as follows: (A) 83%, (B) 94%, (C) 17%, and (D) 6%.

Figure 4. Enlist™ (E) corn plants 7 d after herbicide application with (A) Enlist Duo™ formulation (EDH) only, (B) EDH plus formulated acetochlor, or (C) formulated acetochlor only. The tank mixture of EDH and formulated acetochlor (B) injured E corn (black arrows), but applications of EDH (A) or formulated acetochlor (C) alone did not result in injury. Uptake of radiolabeled 2,4-D (averaged over all time points, ranging from 1 to 24 HAA) increased from 30% with EDH alone to 42% when formulated acetochlor was included.

smaller amount of ^{14}C material was located throughout the remainder of the plant (Figure 3C and D; 17% and 6%), mainly in the stem around the point of attachment of the treated leaf.

The natural tolerance mechanism of corn (Roberts 1998) may explain the lack of any differences observed in translocation of 2,4-D between E and NT corn, in contrast to significant differences noted previously in E and NT soybean varieties (Skelton et al. 2013, 2014a), since non–AAD transformed soybeans are not tolerant to 2,4-D. For example, translocation of 2,4-D free acid away from the treated leaf via the phloem is likely precluded in both corn hybrids by the rapid detoxification of 2,4-D and sequestration of polar metabolites in vacuoles or cell walls (Chkanikov et al. 1976; Feung et al. 1975; Montgomery et al. 1971; Robertson and Kirkwood 1970; Schroder and Collins 2002).

Influence of Tank Mixing Formulated Acetochlor on 2,4-D Uptake and E Corn Injury. Injury from 2,4-D (at a 1X rate) to E corn is not frequently observed under field conditions (Ruen et al. 2017) as compared with E soybean (Skelton et al. 2013), and foliar 2,4-D uptake levels in corn (Figure 2) are lower than in soybean (Skelton et al. 2014a). However, other herbicides with soil-applied residual activity might be included in tank-mix combination with EDH for added residual weed control in E corn. A whole-plant study was conducted to test the hypothesis that injury to E corn may result from increased 2,4-D uptake when EDH and formulated acetochlor are tank mixed (Table 1).

Following application of an EDH-formulated acetochlor tank mix, formation of necrotic lesions near the leaf tip and outermost leaf margins developed within 24 HAA and proceeded to develop farther down the leaf blade (Figure 4; symptoms 7 d after treatment are depicted). The form and extent of foliar injury resemble the rapid development of necrotic spots often observed in mature E soybean leaves following EDH treatment (Skelton et al. 2013) but are atypical of synthetic auxin herbicides (Grossman et al. 2010; Peterson et al. 2016). Uptake of 2,4-D differed between the two treatments ($P \leq 0.0001$) when averaged over all time points (ranging from 1 to 24 HAA), which associates with development of foliar injury symptoms (Figure 4). When EDH was applied alone, the mean uptake of 2,4-D was 30% but increased to 42% when adding formulated acetochlor, which

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contrasts with the lack of ADJ and/or glyphosate influence on 2,4-D uptake (Table 2).

Elevated cellular concentrations of 2,4-D following increased foliar absorption and a subsequent accumulation of reactive oxygen species (Grossman et al. 2010) may have contributed to the injury induced by EDH plus formulated acetochlor in E corn (Figure 4). Cellular AAD-1 enzyme activity may become rapidly overwhelmed by increasing 2,4-D concentrations, due to the lower binding affinity (higher K_m) and lower in vitro activity (lower k_{cat}) of AAD-1 with 2,4-D as substrate relative to AAD-12 in E soybean (Wright et al. 2010). This theory could potentially account for the development of foliar injury symptoms in response to a relatively small increase of 2,4-D uptake in E corn (Figure 4) compared with E soybean (Skelton et al. 2013, 2014a).

Excised-Leaf Assays

Metabolism of 2,4-D in E and NT Corn. The hypothesis that nonphytotoxic metabolites form at a slower rate in NT corn than in E corn was tested using methods previously developed for herbicide metabolism studies with excised leaves (Kreuz and Fonne-Pfister 1992; Ma et al. 2013). The overall amount of 2,4-D metabolized (M_{max}) differed between the two corn hybrids ($P = 0.005$), but the rates of metabolism (M_{50}) when averaged among all herbicide treatments did not ($P = 0.62$; Table 4). The M_{max} of 2,4-D in E corn was greater (100%) compared with NT corn (83.9%). Metabolism of 2,4-D was equal in both hybrids at 1 and 3 HAA, but E corn displayed between 11% and 13% greater metabolism of 2,4-D at each successive time point (Figure 5). The addition of glyphosate did not affect either M_{max} ($P = 0.11$) or M_{50} ($P = 0.07$) parameters (Table 4).

Nonextractable ^{14}C-labeled material measured between the two hybrids (40% in E corn compared with 41% in NT corn) was not different when averaged across all time points in the metabolism study ($P = 0.75$; unpublished data). However, plants treated with 2,4-D plus glyphosate accumulated more nonextractable ^{14}C material (47%) compared with the 2,4-D–only treatment (34%). This difference can be explained statistically through a three-way (hybrid by treatment by hours) interaction ($P = 0.001$). For example, NT corn treated with only 2,4-D displayed lower nonextractable ^{14}C material at all time points (except for 24 HAA) compared with NT corn treated with 2,4-D plus glyphosate, as well as in comparison with both treatments in E corn. Nonextractable ^{14}C material was not different between 2,4-D only or 2,4-D plus glyphosate at any time point in E corn. Although no further attempts were made to solubilize or characterize these nonextractable compounds in E corn leaves, previous research using extraction under acidic or basic hydrolytic conditions has provided insights into the nature of conjugates formed from 2,4-D *in planta* (Hamburg et al. 2001; Zhou et al. 2016).

Table 4. Nonlinear regression parameters for 2,4-D metabolism from excised-leaf assays with Enlist™ (E) and non–AAD-1 transformed (NT) isogenic corn hybrids.

		M_{max}[a]	M_{50}[b]
Hybrid	Treatment	% Metabolized[c,d]	Hours[e]
E		100 w	2.3 z
NT		83.8 x	2.0 z
	2,4-D only	97.4 y	2.7 z
	2,4-D + glyphosate	87.1 y	1.8 z

[a] Parameter corresponding to modeled maximum amount of 2,4-D metabolized of treatment.

[b] Parameter corresponding to time (h) for treatment to reach 50% M_{max}.

[c] Values followed by the same letter within each experiment are not significantly different as determined by Fisher's LSD test ($\alpha = 0.05$).

[d] M_{max} values for hybrid were significantly different ($P = 0.005$), but herbicide treatments were not significantly different ($P = 0.093$).

[e] M_{50} values for hybrid and herbicide treatments were not significantly different ($P = 0.62$ and $P = 0.11$, respectively).

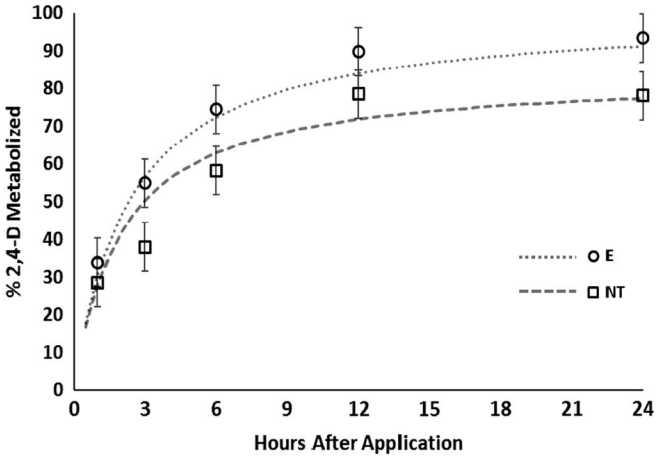

Figure 5. Metabolism of 2,4-D averaged among all herbicide treatments in Enlist™ (E) and non–AAD-1 transformed (NT) corn hybrids from the excised-leaf assay. Symbols represent the mean amount of 2,4-D metabolized; vertical bars represent the standard error of the nonlinear regression model at each time point. Treatment means are the result of combining data from two separate experiments with three replications per individual treatment.

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Metabolism of DCP in E and NT Corn. Metabolism of DCP (when supplied as substrate) to polar metabolites in E and NT corn leaves was qualitatively similar, and DCP was not detectable at any time point in either hybrid (Figure 6A and B). This finding implies that DCP is rapidly metabolized by corn and does not accumulate during the time course examined. By 24 HAA, both hybrids formed four distinct metabolites (Figure 6A and B; retention times are approximately 4, 5, 6.5, and 11 min), but

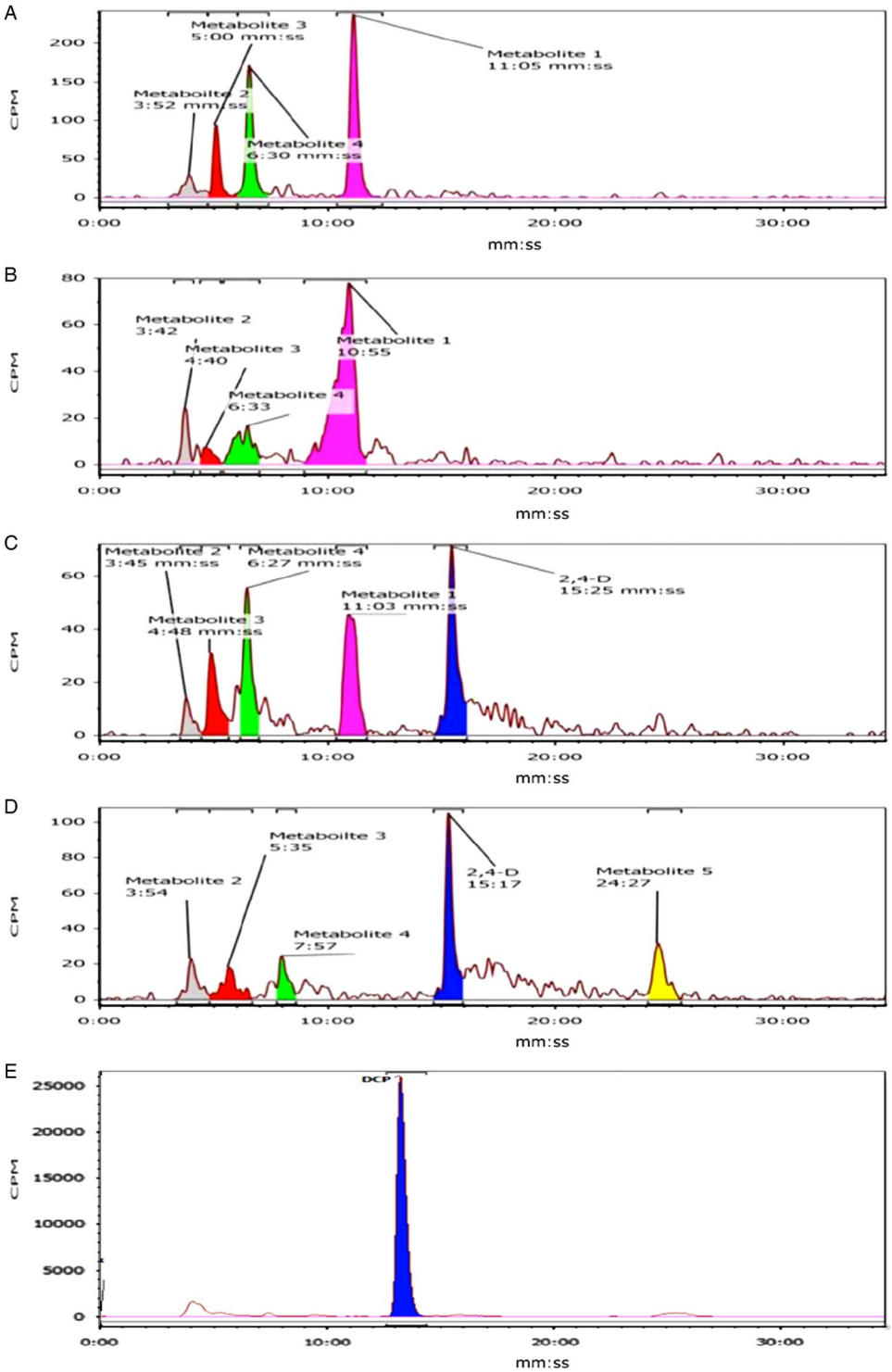

Figure 6. Comparative high-pressure liquid chromatography radiochromatograms depicting [^{14}C]DCP and [^{14}C]2,4-D metabolism in Enlist™ (E) or non–AAD-1 transformed (NT) excised corn leaves at 24 HAA. (A) DCP metabolism by E corn, (B) DCP metabolism by NT corn, (C) 2,4-D metabolism by E corn, and (D) 2,4-D metabolism by NT corn. Retention time of DCP is 13.5 min (E) and 2,4-D is about 15 min under these conditions. Abbreviation: CPM, counts per minute.

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only one metabolite (11 min) was specific to DCP metabolism (compare Figure 6B and D). Rapid metabolism of DCP matches the results from previous studies in wheat (*Triticum aestivum* L.), soybean, tobacco (*Nicotiana tabacum* L.), and several other edible dicots (Laurent et al. 2006, 2007; Pascal-Lorber et al. 2003) and clearly indicates that, when DCP is applied as a nonherbicidal substrate, E and NT corn metabolize DCP similarly by endogenous plant enzymes.

E corn treated with 2,4-D formed the metabolite specific to DCP metabolism (Figure 6A and C; approximately 11 min). Rapid metabolism of 2,4-D to DCP is catalyzed by the AAD-1 enzyme in E corn (Wright et al. 2010). DCP is then rapidly turned over and polar metabolite(s) of DCP are formed when E corn leaves are supplied with 2,4-D as substrate (compare Figure 6A and C). However, the metabolite(s) peak at approximately 11 min was not detected when NT corn leaves were supplied with 2,4-D (Figure 6D), consistent with an inability to form DCP from parent 2,4-D. Three polar metabolites (Figure 6C and D; approximately 4, 5, and 6.5 min) were detected in E and NT corn treated with 2,4-D; a relatively nonpolar metabolite specific to 2,4-D metabolism by NT corn was also detected (Figure 6D; approximately 24 min). These first three peaks may contain ring-hydroxylated and/or glycosylated metabolites generated by endogenous 2,4-D metabolic pathways previously characterized in corn, wheat, soybean, tobacco, and several other dicot and monocot plants (Chkanikov et al. 1976; Feung et al. 1975; Hamburg et al. 2001; Montgomery et al. 1971; Robertson and Kirkwood 1970; Roberts 1998; Schroder and Collins 2002; Zhou et al. 2016), but were not characterized further.

E corn metabolized more 2,4-D than NT corn, and glyphosate did not alter 2,4-D metabolism in either hybrid (Table 4). The excised-leaf assay generated new metabolic information, which provided mechanistic insight into field studies showing that E corn can tolerate higher rates and later POST application timings of 2,4-D than NT corn hybrids (Wright et al. 2010). Accumulation of nonextractable radiolabeled material (i.e., bound residues) was not different between hybrids. This finding correlates with the equivalent amounts of translocation between hybrids determined by the whole-plant study (Table 3). Rapid detoxification of 2,4-D to immobile, polar metabolites may inhibit translocation to meristematic tissues in both hybrids and may enable storage or sequestration of metabolite(s) in the treated leaves via phase III transport-detoxification mechanisms (Hatzios 2005; Van Eerd et al. 2003). Initial metabolites rapidly formed during 2,4-D metabolism by E and NT corn hybrids (Figure 6C and D) are different due to the conversion of 2,4-D to DCP by AAD-1 in E corn leaves. DCP does not accumulate but is rapidly metabolized to a polar metabolite (Figure 6C; approximately 11 min) in E corn that is not detectable when 2,4-D is supplied as substrate in NT corn (Figure 6D). Recent residue analyses have determined the long-term metabolic fate of 2,4-D choline in various plant tissues and grain beyond 24 HAA in E corn (Zhou et al. 2016).

Glyphosate does not alter 2,4-D uptake or metabolism in corn, and EDH resulted in relatively slower uptake of 2,4-D compared with previous findings in E soybean (Skelton et al. 2013, 2014a). The relatively lower amount and rate of 2,4-D uptake in E corn, in conjunction with endogenous mechanisms for 2,4-D detoxification, may decrease the likelihood of corn injury under field conditions. This theory is corroborated by tolerance of E corn lines of up to 3.4 kg ae ha^{-1} of 2,4-D per growing season (Wright et al. 2010). However, injury was observed in E corn when 2,4-D uptake increased following a tank-mix application of EDH with formulated acetochlor. Our research did not determine which component(s) of formulated acetochlor contributed to this increase, but the active ingredient, formulation, organic solvent, or a combination of these components may have contributed to the significant increase in 2,4-D uptake and resulting foliar injury. E corn may be more sensitive to a rapid increase in cellular 2,4-D concentrations, since the combination of the AAD-1 enzyme and endogenous enzyme(s) in corn metabolizes 2,4-D less rapidly than the AAD-12 enzyme in E soybean (Skelton et al. 2014b). Alternatively, a component of formulated acetochlor and/or the interaction of acetochlor with events triggered by the mechanism of 2,4-D action (Grossman et al. 2010) may have induced injury instead of an increase in 2,4-D uptake.

The *aad-1* transgene in E corn enhances natural 2,4-D tolerance and confers AOPP resistance (Wright et al. 2010). These novel traits increase the number of effective sites of action in corn for POST control of dicot and grass weeds, especially glyphosate-resistant grasses (Mueller et al. 2011; Perez-Jones et al. 2005; Vila Aiub et al. 2007). Tank mixtures of 2,4-D with other herbicide chemistries and sequential management programs are necessary to minimize the risk of selecting for 2,4-D–resistant

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weeds in E crops (Peterson et al. 2016) and to provide soil residual activity for extended weed control. Further research should focus on measuring the amounts and rates of 2,4-D uptake that result from potential tank mixtures with 2,4-D choline and determining their combined effects on herbicide metabolism, crop injury, and weed control, especially when managing glyphosate-resistant weed populations. This complementary research would provide beneficial agronomic information and promote proper stewardship of EDH for integrated weed management programs in corn, soybean, and cotton.

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