

Resistance of Enlist™ (AAD-12) Cotton to Glufosinate

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Enlist™ cotton contains the *aad-12* and *pat* genes that confer resistance to 2,4-D and glufosinate, respectively. Thirty-three field trials were conducted focused on Enlist cotton injury from glufosinate as affected by cotton growth stage, application rate, and single or sequential applications. Maximum injury from a single application of typical 1X (542 g ae ha⁻¹) and 2X use rates was 3 and 13%, respectively, regardless of growth stage. Injury from sequential applications of 1X or 2X rates was equivalent to single applications. Similar injury was observed with four commercial formulations of glufosinate. Cotton yield was never affected by glufosinate. This research demonstrates Enlist™ cotton has robust resistance to glufosinate at rates at least twice the typical use rate when applied once or twice at growth stages ranging from 2 to 12 leaves.

Nomenclature: Glufosinate; 2,4-D; cotton, *Gossypium hirsutum* L.

Key words: Aryloxyalkanoate dioxygenase, DAS-81910-7, herbicide-resistant crops, phosphinothricin acetyltransferase.

Herbicide resistance is a serious problem in the United States and other developed countries and threatens our ability to economically and sustainably produce food and fiber. Biotypes of weeds resistant to 19 of the 26 known herbicide mechanisms of action have been confirmed in the United States, and biotypes resistant to 23 of the mechanisms have been confirmed globally (Heap 2016). In the US Cotton Belt, glyphosate-resistant (GR) Palmer amaranth (*Amaranthus palmeri* S. Wats.) is the most widespread and economically damaging resistant weed (Culpepper et al. 2010; Ward et al. 2013), although there are also problems with GR biotypes of common ragweed (*Ambrosia artemisiifolia* L.), giant ragweed (*Ambrosia trifida* L.), tall waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer], goosegrass [*Eleusine indica* (L.) Gaertn.], horseweed [*Conyza canadensis* (L.) Cronq.], johnsongrass [*Sorghum halepense* (L.) Pers.], Italian ryegrass [*Lolium perenne* L. spp. *multiflorum* (Lam.) Husnot], and kochia [*Kochia scoparia* (L.) Schrad.] (Heap 2016). Weed biotypes resistant to acetyl CoA carboxylase inhibitors, acetolactate synthase (ALS) inhibitors, microtubule assembly inhibitors, nucleic acid inhibitors,

protoporphyrinogen oxidase inhibitors, photosystem I electron diverters, and photosystem II electron transport inhibitors are also present in the Cotton Belt (Heap 2016).

As incidences of resistance continue to increase, growers have fewer options to control weeds. Residual herbicides are consistently recommended in cotton weed management programs, especially for Palmer amaranth (Culpepper 2016; Wilson et al. 2011; York 2016). However, POST topically applied herbicides are needed in these programs for the control of emerged Palmer amaranth plants, as PRE herbicides alone will not adequately control this weed (Culpepper and York 1997; Keeling et al. 1991; Riar et al. 2011; Whitaker et al. 2011). Currently available POST options to control GR Palmer amaranth in cotton are limited to pyriithiobac, trifloxysulfuron, and glufosinate. Pyriithiobac and trifloxysulfuron are ALS inhibitors, and multiple resistance to both glyphosate and ALS inhibitors is common (Heap 2016, Poirier et al. 2014, Sosnoskie et al. 2011).

Glufosinate can control Palmer amaranth (Corbett et al. 2004), but control is often inadequate in a

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glufosinate-only program (Merchant et al. 2014; Whitaker et al. 2011; Wilson et al. 2007). However, if glufosinate is applied in a timely manner and used in conjunction with residual soil-applied herbicides, Palmer amaranth can be controlled well (Cahoon et al. 2015; Everman et al. 2009; Gardner et al. 2006). With the advent of GR Palmer amaranth, cotton growers are increasingly relying on glufosinate applied multiple times per season (Sosnoskie and Culpepper 2014). Increased reliance on glufosinate has raised concerns over the increased selection pressure for glufosinate-resistant biotypes (Burgos et al. 2015; Jalaludin et al. 2015; York 2016).

Widespread evolution of herbicide-resistant weed biotypes has created an imperative for alternative weed management strategies, including additional herbicide mechanisms of action (Hay 1999). Unfortunately, herbicides with new mechanisms of action are not being developed (Duke 2012). Crops that contain traits conferring resistance to existing herbicides previously not used in the crop, or resistance to multiple herbicide mechanisms of action, can expand the utility of existing herbicide technologies and enable new management solutions (Beckie 2014; Green and Owen 2011; Green 2012).

Enlist™ cotton (DAS-81910-7) was developed using *Agrobacterium*-mediated transformation to stably insert the transformation plasmid pDAB4468 into a conventional cotton variety. The inserted binary pDAB4468 plasmid contains two gene cassettes. The first cassette contains the aryloxyalkanoate dioxygenase-12 (*aad-12*) gene, and the second cassette contains the phosphinothricin acetyltransferase (*pat*) gene, each under the control of constitutive promoters (USDA-APHIS 2013). The *aad-12* gene and the *pat* gene simultaneously encode for the aryloxyalkanoate dioxygenase-12 and phosphinothricin acetyltransferase enzymes, which confer resistance to the herbicides 2,4-D and glufosinate, respectively (USFDA 2014). The original transformant was subsequently used in a standard plant breeding regimen to produce the experimental varieties utilized in the experiments reported herein.

There are no currently published data on resistance of Enlist cotton to glufosinate. Our objective was to determine the resistance to glufosinate conferred by the Enlist cotton trait as affected by glufosinate rate, single or sequential glufosinate application, and cotton growth stage at application.

Materials and Methods

Thirty-three field experiments were conducted across the major cotton-producing areas of the United States in 2010 through 2013 and in 2016 (Table 1). An experimental cotton variety containing the Enlist cotton event of interest (DAS-81910-7) was planted at the rate of 13 seeds per meter of row into weed-free conventionally tilled seedbeds. Plots were four rows wide (91- to 97-cm row spacing) by 6 m long. Treatments were arranged in a randomized complete block design and replicated four times. Each location received a PRE herbicide, with herbicide selection based on historic weed populations, soil type, and local practice. After cotton emergence, plots were kept weed-free through the use of mechanical cultivation and removal of weeds by hand. Other production practices, including fertilization, insect control, plant growth regulator application, and defoliation, were performed according to local practice. Treatment variables (glufosinate rate, timing of application, formulation of glufosinate) were applied to the center two rows of each plot, leaving a two-row unsprayed buffer between treatments.

Treatments in 2010 consisted of glufosinate-ammonium (Liberty® 280 SL herbicide, Bayer CropScience LP, Research Triangle Park, NC 27709) applied once to 6- to 8-leaf cotton at rates of 542, 1,084, and 2,168 g ae ha⁻¹. The typical (1 ×) glufosinate use rate for cotton is 542 g ha⁻¹ (Anonymous 2016). Treatments in 2011 included a single application of glufosinate at 542 and 1,084 g ha⁻¹ to 6- to 8-leaf cotton or 10- to 12-leaf cotton, and sequential applications of the same rates at each growth stage. The time interval between 6- to 8-leaf and 10- to 12-leaf applications ranged from 11 to 16 d, depending upon location. Treatments in 2012 and 2013 were similar to those in 2011, except that a third rate of glufosinate, 804 g ha⁻¹, was included during the single and sequential application timings.

An experiment at four locations in 2016 (Table 1) focused on Enlist cotton response to four commercial products containing 24.5% (w/v) glufosinate-ammonium. Commercial brands included Cheetah® herbicide (Nufarm, Inc, 11901 S. Austin Ave., Alsip, IL 60803), Interline® herbicide (United Phosphorus, Inc., 630 Freedom Business Center, Suite 402, King of Prussia, PA 19406), Kong® Glufosinate 280 herbicide (Solera ATO, LLC, 12230 E. Del Norte,

Table 1. Location, soil type, and planting and harvest dates of field experiments.

| Year | Location | Coordinates | Soil series | Planting date | Harvest date | |
|---------------------|---------------------|-----------------------|-------------------------------------|--------------------|-----------------|--------|
| 2010 | Headland, AL | 31.38°N, 85.31°W | Lucy loamy sand ^a | 20-May | 28-Oct | |
| | Jacksonville, AR | 34.80°N, 92.09°W | Rilla silt loam ^b | 2-Jun | 18-Oct | |
| | Fresno, CA | 36.72°N, 119.93°W | Pachappa loam ^c | 25-May | NH ⁿ | |
| | Attapulgus, GA | 30.76°N, 84.48°W | Lucy loamy sand | 3-Jun | NH | |
| | Brinson, GA | 30.97°N, 84.70°W | Blanton loamy sand ^d | 25-May | 29-Oct | |
| | Winsboro, LA | 32.14°N, 91.69°W | Egypt silt loam ^e | 24-May | 29-Sep | |
| | Greenville, MS | 33.27°N, 91.03°W | Commerce silt loam ^f | 24-May | 23-Aug | |
| | Verona, MS | 34.17°N, 88.74°W | Leeper fine sandy loam ^g | 8-Jun | 17-Oct | |
| | Lewiston, NC | 36.14°N, 77.18°W | Rains sandy loam ^h | 1-Jun | 29-Oct | |
| | Elko, SC | 33.41°N, 81.33°W | Wagram sand ⁱ | 8-Jun | 9-Oct | |
| | Memphis, TN | 35.12°N, 89.81°W | Waverly silt loam ^j | 28-May | 8-Nov | |
| | 2011 | North Little Rock, AR | 34.80°N, 92.09°W | Rilla silt loam | 31-May | 26-Oct |
| | | Brinson, GA | 30.97°N, 84.70°W | Blanton loamy sand | 18-May | 12-Oct |
| | | Greenville, MS | 33.27°N, 91.03°W | Commerce silt loam | 19-May | 14-Oct |
| Jackson Springs, NC | | 35.19°N, 79.69°W | Candor sand ^k | 27-May | 10-Oct | |
| Halfway, TX | | 34.16°N, 101.95°W | Olton clay loam ^l | 19-May | NH | |
| Lubbock, TX | | 33.70°N, 101.81°W | Olton clay loam | 18-May | NH | |
| Memphis, TN | | 35.12°N, 89.81°W | Waverly silt loam | 1-Jun | 25-Oct | |
| 2012 | | Brinson, GA | 30.97°N, 84.70°W | Blanton loamy sand | 9-May | 16-Oct |
| | Winnboro, LA | 32.14°N, 91.69°W | Egypt silt loam | 15-May | 22-Oct | |
| | Greenville, MS | 33.27°N, 91.02°W | Commerce silt loam | 10-May | 10-Oct | |
| | Verona, MS | 34.17°N, 88.74°W | Leeper fine sandy loam | 16-May | 11-Oct | |
| | Jackson Springs, NC | 35.67°N, 78.51°W | Candor sand | 23-May | 12-Oct | |
| | Hale Center, TX | 34.15°N, 101.95°W | Pullman clay loam ^m | 18-May | 30-Oct | |
| | Elko, SC | 33.41°N, 81.33°W | Wagram sand | 9-May | 30-Nov | |
| | 2013 | Brinson, GA | 30.97°N, 84.70°W | Blanton loamy sand | 22-May | 28-Oct |
| Greenville, MS | | 33.27°N, 91.02°W | Commerce silt loam | 29-May | 9-Oct | |
| Verona, MS | | 34.17°N, 88.74°W | Leeper fine sandy loam | 29-May | 24-Oct | |
| Jackson Springs, NC | | 35.20°N, 79.69°W | Candor sand | 29-May | 8-Nov | |
| 2016 | Brinson, GA | 30.97°N, 84.70°W | Blanton loamy sand | 04-May | 29-Sep | |
| | Greenville, MS | 33.27°N, 91.02°W | Commerce silt loam | 06-May | 23-Sep | |
| | Verona, MS | 34.17°N, 88.74°W | Leeper fine sandy loam | 11-May | 21-Sep | |
| | Elko, SC | 33.41°N, 81.33°W | Wagram sand | 26-May | 13-Oct | |

^a Loamy, kaolinitic, thermic Arenic Kandiudults

^b Fine-silty, mixed, active, thermic Typic Hapludalfs

^c Coarse-loamy, mixed, active, thermic Mollic Haploxeralfs

^d Loamy, siliceous, semiactive, thermic Grossarenic Paleudults

^e Fine-silty, mixed, active, thermic Aquic Glossudalfs

^f Fine-silty, mixed, superactive, nonacid, thermic Fluvaquentic Endoaquepts

^g Fine, smectitic, nonacid, thermic Vertic Epiaquepts

^h Fine-loamy, siliceous, semiactive, thermic Typic Paleaquults

ⁱ Loamy, kaolinitic, thermic Arenic Kandiudults

^j Coarse-silty, mixed, active, acid, thermic Fluvaquentic Endoaquepts

^k Sandy, kaolinitic, thermic Grossarenic Kandiudults

^l Fine, mixed, superactive, thermic Aridic Paleustolls

^m Fine, mixed, superactive, thermic Torriertic Paleustolls

ⁿ Abbreviation: NH, not harvested.

Yuma, AZ 85367), and Liberty 280 SL. Each herbicide was applied to 2- to 4-leaf cotton at 542 and 1,084 g ha⁻¹ and repeated at the same rates 12 to 15 d later, when cotton had six to eight leaves.

In all trials, glufosinate was applied using CO₂-pressurized backpack sprayers delivering a spray volume of 140 L ha⁻¹. Sprayers were equipped with TeeJet® Drift Guard flat-fan spray tips in 2010

through 2012, and with TeeJet Air Induction Extended Range flat-fan tips in 2013 and 2016 (TeeJet Technologies, Glendale Heights, IL). Crop injury was estimated visually as described by Frans et al. (1986) at 3, 7, and 14 d after application (DAA) using a scale of 0% (no injury) to 100% (complete crop death). Foliar chlorosis and necrosis and growth reduction were considered when estimating injury. Plots were mechanically harvested to determine seed cotton yield, and data were converted to lint yield assuming a 38% gin turnout.

Statistical Analysis. A linear mixed model (ANOVA) was fit using the **lme4** package in R version 3.2.1 (The R Foundation for Statistical Computing, c/o Institute for Statistics and Mathematics, Wirtschaftsuniversität Wien, Welthandelsplatz, 1020 Vienna, Austria). In the model, herbicide treatment was the fixed effect and trial location, year, and replication were random effects. To satisfy ANOVA assumptions, visual crop injury estimates and yields were subjected to a Box-Cox transformation (Box and Cox 1964) using the **car** package in R, which determined the power transformation that maximized the log likelihood function for each response variable. However, we present back-transformed data, with means rounded to whole numbers and means separations based on transformed data. Where the ANOVA test indicated that treatment effects were significant, means were separated at $\alpha = 0.05$ using Tukey's honest significant difference test. Lint yields of glufosinate-treated cotton were compared to the yield of nontreated cotton using Dunnett's procedure (Dunnett 1955).

Results and Discussion

In 2010, Glufosinate at the 1 × rate of 542 g ha⁻¹ applied to 6- to 8-leaf cotton injured the crop only 3% at 3 DAA, and no injury was perceptible at 14 DAA (Table 2). Glufosinate at 2 × and 4 × rates of 1,084 and 2,168 g ha⁻¹ injured cotton 7% and 13%, respectively, at 3 DAA. Injury decreased over time, and only 2% injury was noted with the 4 × rate at 14 DAA. In all trials in 2010 and subsequent years, injury was expressed primarily as foliar necrosis along with minor chlorosis. No reduction in cotton growth was noted. Cotton lint yield was unaffected by glufosinate.

Table 2. Injury and yield of Enlist cotton following a single POST application of glufosinate at the 6- to 8-leaf growth stage in 2010.^a

| Glufosinate rate | Injury | | | Lint yield ^c |
|--------------------|--------------------|-------|--------|-------------------------|
| | 3 DAA ^b | 7 DAA | 14 DAA | |
| g ha ⁻¹ | % | | | kg ha ⁻¹ |
| 0 | | | | 965 |
| 542 | 3 a | 2 a | 0 b | 970 |
| 1,084 | 7 b | 4 b | 0 b | 1,000 |
| 2,168 | 13 c | 10 c | 2 a | 970 |

^a Data for injury and yield averaged over 11 and 9 locations, respectively. Means within a column followed by the same letter are not different according to Tukey's honest significant difference test ($\alpha = 0.05$).

^b Abbreviation: DAA, days after application.

^c Yield of glufosinate-treated cotton did not differ from yield of nontreated cotton according to Dunnett's procedure ($\alpha = 0.05$).

In 2011, glufosinate at 542 g ha⁻¹ injured 6- to 8-leaf cotton and 10- to 12-leaf cotton 1% to 2% at 3 DAA, while glufosinate at 1,084 g ha⁻¹ caused 5% to 6% injury at 3 DAA (Table 3). Similar injury was noted when glufosinate was applied to 6- to 8-leaf cotton and 10- to 12-leaf cotton. Injury from sequential applications was initially no greater than was injury from the 10- to 12-leaf application. However, greater injury was noted with sequential applications 7 d after the second application compared with that observed with one application to 10- to 12-leaf cotton. However, injury 7 DAA was only 10%. No injury was observed with single or sequential applications of 1 × and 2 × rates at 14 DAA. No glufosinate treatment impacted cotton yield in 2011.

Injury observed in 2012 and 2013 was similar to that observed in 2011. Glufosinate at 542, 804, and 1,084 g ha⁻¹ injured cotton 3%, 5% to 6%, and 7% to 9%, respectively, at 3 DAA (Table 3). The crop was injured only 3% by the highest rate of glufosinate at 14 DAA. Glufosinate applied sequentially to 6- to 8-leaf and 10- to 12-leaf cotton was no more injurious than was glufosinate applied only to 10- to 12-leaf cotton. Regardless of application rate, growth stage at application, or number of applications, cotton yield was not impacted by glufosinate.

No differences in cotton injury were noted among the four brands of glufosinate applied at 542 g ha⁻¹ to 2- to 4-leaf cotton or 6- to 8-leaf cotton at 3, 7, or 14 DAA (Table 4). When glufosinate was applied at 1,084 g ha⁻¹ to 2- to 4-leaf cotton, injury was 8% or

Table 3. Injury and yield of Enlist cotton following single and sequential POST applications of glufosinate in 2011, 2012, and 2013.^a

| Cotton growth stage at application | Glufosinate rate | Injury ^b | | | | | | Lint yield ^d | |
|---|------------------|---------------------|------|-----|-----------|-----|---------------------|-------------------------|-----------|
| | | 2011 | | | 2012–2013 | | | 2011 | 2012–2013 |
| | | DAA ^c | | | DAA | | | | |
| g ha ⁻¹ | 3 | 7 | 14 | 3 | 7 | 14 | kg ha ⁻¹ | | |
| | 0 | % | | | | | | kg ha ⁻¹ | |
| 6- to 8-leaf | 542 | 1 b | 1 c | 0 a | 3 c | 2 a | 1 c | 1,220 | 1,535 |
| 6- to 8-leaf | 804 | | | | 5 abc | 4 a | 2 b | 1,265 | 1,530 |
| 6- to 8-leaf | 1,084 | 5 ab | 2 b | 1 a | 9 a | 6 a | 3 ab | 1,135 | 1,525 |
| 10- to 12-leaf | 542 | 2 ab | 2 b | 0 a | 3 c | 2 a | 1 c | 1,130 | 1,545 |
| 10- to 12-leaf | 804 | | | | 6 abc | 4 a | 1 c | | 1,525 |
| 10- to 12-leaf | 1,084 | 6 a | 4 b | 1 a | 7 abc | 6 a | 3 ab | 1,145 | 1,505 |
| 6- to 8- fb ^c 10- to 12-leaf | 542 fb 542 | 2 ab | 10 a | 0 a | 4 bc | 2 a | 1 c | 1,150 | 1,485 |
| 6- to 8- fb 10- to 12-leaf | 804 fb 804 | | | | 7 abc | 5 a | 2 b | | 1,530 |
| 6- to 8- fb 10- to 12-leaf | 1,084 fb 1,084 | 5 ab | 10 a | 1 a | 8 ab | 6 a | 4 a | 1,140 | 1,520 |

^a Injury and yield data averaged over 7 and 5 locations, respectively, in 2011. Injury and yield data averaged over 11 locations in 2012 and 2013. Means within a column followed by the same letter are not different according to Tukey's honest significant difference test ($\alpha = 0.05$).

^b Injury for sequential applications recorded following the second application.

^c Abbreviations: DAA, days after application; fb, followed by.

^d Yield of glufosinate-treated cotton did not differ from yield of nontreated cotton according to Dunnett's procedure ($\alpha = 0.05$).

less. Significantly higher levels of injury were noted with Interline and Liberty than were with Kong at 3 DAA, and at 7 DAA cotton treated with Interline had significantly more injury than did that treated with Kong. The same response was noted 3 DAA to 6- to 8-leaf cotton, with no significant differences between glufosinate brands except that more injury

was observed with Interline than with Kong. No differences among glufosinate brands were noted 7 or 14 DAA to 6- to 8-leaf cotton. No glufosinate treatment reduced cotton yield compared with the no-glufosinate treatment.

This research demonstrates that Enlist cotton has robust resistance to glufosinate at rates at least twice the

Table 4. Injury and yield of cotton with four brands of glufosinate applied at the 2- to 4-leaf growth stage and re-applied at the 6- to 8-leaf growth stage in 2016.^a

| Glufosinate brand | Rate | Cotton injury | | | | | | Lint yield ^b |
|-------------------|--------------------|-------------------------------------|------|------|-------------------------------------|-----|-----|-------------------------|
| | | days after 2- to 4-leaf application | | | days after 6- to 8-leaf application | | | |
| | | 3 | 7 | 14 | 3 | 7 | 14 | |
| | g ha ⁻¹ | % | | | | | | kg ha ⁻¹ |
| No glufosinate | | | | | | | | 1,148 |
| Cheetah | 542 | 3 bc | 2 bc | 1 ab | 4 ab | 3 a | 0 a | 1,198 |
| Cheetah | 1,084 | 5 ab | 4 ab | 1 ab | 7 a | 4 a | 1 a | 1,167 |
| Interline | 542 | 3 bc | 2 bc | 2 ab | 3 ab | 2 a | 0 a | 1,229 |
| Interline | 1,084 | 8 a | 6 a | 3 a | 7 a | 5 a | 1 a | 1,151 |
| Kong | 542 | 1 c | 0 c | 0 b | 2 b | 2 a | 1 a | 1,210 |
| Kong | 1,084 | 3 bc | 1 bc | 0 b | 3 ab | 4 a | 1 a | 1,149 |
| Liberty | 542 | 4 ab | 2 bc | 0 b | 3 ab | 3 a | 0 a | 1,164 |
| Liberty | 1,084 | 8 a | 5 ab | 2 ab | 7 a | 5 a | 1 a | 1,176 |

^a Data averaged over four locations. Means within a column followed by the same letter are not different according to Tukey's honest significant difference test ($\alpha = 0.05$).

^b Yield of glufosinate-treated cotton did not differ from yield of nontreated cotton according to Dunnett's procedure ($\alpha = 0.05$).

typical use rate when applied once or twice at growth stages ranging from 2- to 12-leaf. Resistance was similar to that described in previous research with LibertyLink and GlyTol + LibertyLink cotton (Dodds et al. 2015; Irby et al. 2013; Sweeney and Jones 2014; Wallace et al. 2011). In other work (Richburg et al. 2015; Dow AgroSciences internal research reports, unpublished data), Enlist cotton has been shown to be very tolerant to 2,4-D applied topically. With resistance to both glufosinate and 2,4-D, Enlist cotton will give growers more options for controlling GR weeds. Mixtures of glufosinate plus 2,4-D will improve consistency of weed control (Chahal and Johnson 2012; Craigmyle et al. 2013; Johnson et al. 2010; Merchant et al. 2013, 2014; Steckel et al. 2006). Additionally, co-application of glufosinate and 2,4-D will delay evolution of resistance to both herbicides (Diggle et al. 2003; Powles et al. 1997).

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