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# Response of Aryloxyalkanoate Dioxygenase-12 Transformed Soybean Yield Components to Postemergence 2,4-D

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New trait technology incorporating 2,4-dichlorophenoxyacetic acid (2,4-D) resistance in soybean provides an alternative method to control weeds. However, the effect of postemergence treatments of 2,4-D on aryloxyalkanoate dioxygenase-12 (AAD-12) soybean on injury and yield components has not been reported. Our objectives were to characterize the effect of 2,4-D (dimethylamine salt) rates (0, 1,120, and 2,240 g ae ha$^{-1}$) and soybean growth stage (V5, R2, or V5 followed by R2) on AAD-12 soybean injury and yield components. Less than 3% soybean injury was observed when 2,240 g ha$^{-1}$ of 2,4-D was applied to R2 soybean, and less than 1% soybean injury was caused by 1,120 g ha$^{-1}$ of 2,4-D. Seed yield, seed mass, pod number, seed number, seed per pod, reproductive node number, pods per reproductive node, node number, and percent reproductive nodes were not affected by 2,4-D treatments when applied at the V5, R2, or the V5 followed by R2 soybean growth stage. This research demonstrates that soybean transformed with AAD-12 can tolerate foliar applications of 2,4-D at rates up to 2,240 g ha$^{-1}$ with no effect on soybean grain yield components.
**Nomenclature:** 2,4-D (dimethylamine salt); *Glycine max* (L.) Merr., soybean.
**Key words:** 2,4-dichlorophenoxyacetic acid, AAD-12 soybean, Enlist, soybean injury, transgenic soybean, yield components.

The introduction of aryloxyalkanoate dioxygenase-12 (AAD-12) soybean will provide preemergence and postemergence resistance to 2,4-D (Wright et al. 2010), thus allowing 2,4-D to be used to control broadleaf weeds in soybean. The AAD-12 protein was isolated from the soil bacteria *Delftia acidovorans* and can metabolize 2,4-D, triclopyr, and fluroxypyr by a rapid, single step, metabolic detoxification mediated by an Fe(II)/α-ketoglutarate-dependent dioxygenase (Wright et al. 2010). Successful grower adoption of herbicide-resistant, transgenic crops relies on preceding research that demonstrates the associated herbicide applications will allow for efficacious weed control and safe application of the herbicide to the transformed crop. More specifically, the research should provide a characterization of any potential negative effects on the economic value of the crop (e.g., grain or forage yield) that might result from the herbicide applications.

2,4-D–sensitive soybean plants exposed to 2,4-D at rates as low as 1% of common commercial application rates typically express injury symptoms such as leaf crinkling, bubbling, puckering, strapping, and epinasty. Visual injury of 2,4-D–sensitive soybean plants exposed to reduced rates of 2,4-D generally peaked at around 2 wk after treatment (WAT) (Andersen et al. 2004; Kelley et al. 2005; Robinson et al. 2013a). Previous research documented soybean injury ranging from 5%, 8 to 25%, and 30 to 35% when 2,4-D was applied at 11.2, 56, and 112 g ha$^{-1}$, respectively (Andersen et al. 2004; Kelley et al. 2005). Similarly, Robinson et al. (2013a) reported 85, 84, and 62% soybean injury was caused when 1,120 g ha$^{-1}$ 2,4-D was applied to 2,4-D–sensitive soybean at the V2, V5, and R2 soybean growth stages, respectively.

The introduction of foreign genes into the genome of a plant can cause abnormal growth (Dale et al. 1993) and reduce crop yield by either reducing pollen or pollination (Pline et al. 2003) or from a fitness penalty caused by expression of the transgene (Elmore et al. 2001b). Glyphosate treatments on glyphosate-resistant cotton (*Gossypium hirsutum* L.) and corn (*Zea mays* L.) have had adverse effects on pollen viability and reproduction. In cotton, pollen viability was reduced during the first week of flowering to 70% after one application of glyphosate at 1,120 g ae ha$^{-1}$ and to 38% when two applications of 1,120 g ha$^{-1}$ of glyphosate were applied (Pline et al. 2003). A reduction in pollination was linked to reduced boll retention, because anther length was reduced and anthers contained immature, nonviable pollen as a result of glyphosate treatment, which reduced seed set (Jones

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and Snipes 1999; Pline et al. 2003). Similar to cotton, late POST (V8 to V10) glyphosate treatments (1,120 g $ha^{-1}$) to glyphosate-resistant corn reduced pollen viability by 57 to 76% compared to 98 to 99% viability in nontreated corn; however, kernel set and yield were not affected because of the high quantity of pollen produced (Thomas et al. 2004). The reduction in pollination from glyphosate applications was linked to a lower expression of the CP4 EPSPS gene in cotton and corn.

Glyphosate applied to glyphosate-resistant soybean in adverse conditions or when high rates of glyphosate were applied have been reported to affect yield and plant growth. Drought stress following two 1,680 g $ha^{-1}$ glyphosate treatments to glyphosate-resistant soybean reduced yield by 12 to 25% (King et al. 2001). When 2,540 g $ha^{-1}$ glyphosate was applied twice, yield was reduced by 11% in 1 yr of a 3-yr study (Zablotowicz and Reddy 2007), but yield reduction was attributed to reduced nitrogen fixation by symbiotic bacteria. However, other researchers found yield was not reduced from glyphosate treatments on glyphosate-resistant soybean (Delannay et al. 1995; Elmore et al. 2001a; Miller et al. 2008; Nelson and Renner 2001). When glyphosate treatments of 800, 1,200, and 2,400 g $ha^{-1}$ were applied to V2, V4, or V6, second-generation glyphosate-resistant soybean had a reduction in photosynthetic rate; nutrient accumulation; nodule production; leaf area; and root, shoot, and nodule biomass (Zobiole et al. 2012). However, glyphosate treatments of 0 to 2,749 g $ha^{-1}$ applied to R4 glyphosate-resistant soybean did not affect plant height, node number, pod number, or yield (Miller et al. 2008). Although in most cases soybean yield is not reduced following glyphosate treatment, little research has explored its effects on all soybean-yield components during vegetative and reproductive growth. Soybean might be able to compensate for changes in reproductive structures caused by glyphosate in a manner similar to cotton where boll retention was altered, but yield was not changed because the plant compensated for an early boll loss by setting later bolls (Jones and Snipes 1999).

Soybean yield components can be analyzed to identify possible causes of seed yield changes. Yield is affected by seed number and seed mass (primary traits); seed number is a result of pod number and seed per pod (secondary traits). Pod number is then affected by reproductive node number and pod per reproductive node (tertiary traits), and reproductive node number is affected by percent reproductive nodes and node number (quaternary traits) (Board and Modali 2005). Analyzing yield components has been used to determine the effect of planting date (Robinson et al. 2009), comparing old and new cultivars (Kahlon et al. 2011), and determining the effect of dicamba and 2,4-D drift rates on sensitive soybean (Robinson et al. 2013a,b).

As with any new herbicide trait, AAD-12 soybean should be evaluated to determine the effects of 2,4-D treatments on crop resistance and reproductive parameters. By analyzing soybean yield components, AAD-12 soybean can be assessed to determine if any changes in seed yield or yield components occur when exposed to 2,4-D. The objectives of this study were to characterize soybean injury and quantify yield components and seed yield of AAD-12 soybean after foliar 2,4-D exposure.

## Materials and Methods

Field experiments were conducted at the Pinney Purdue Agriculture Center (PPAC) located near Wanatah, IN (41.44328°N, 86.930411°W) in 2009 and 2011 and at the Dow AgroSciences Midwest Research Center (MRC) located near Fowler, IN (40.631881°N, 87.097244°W) in 2008, 2009, and 2011. Soil type at PPAC was a Bourbon sandy loam (coarse-loamy, mixed, mesic, Aquollic Hapludalf) and at MRC was a Drummer silty clay loam (Fine-silty mixed, superactive, mesic Typic Endoaquolls). The event DAS-68416-4 (Dow AgroSciences AAD-12 transformed soybean, Dow AgroSciences, Indianapolis, IN 46286) was planted in 38-cm rows at 420,000 seeds $ha^{-1}$.

Plots were kept weed-free by using preemergence herbicides and by hand weeding. At PPAC, a commercial premix of metribuzin plus chlorimuron-ethyl at 184 g ai $ha^{-1}$ was applied PRE. At MRC in 2008 and 2009, trifluralin (1,400 g ai $ha^{-1}$) plus imazethapyr (70 g ai $ha^{-1}$) were applied PPI, and in 2011 sulfentrazone plus cloransulam-methyl (51.9 g ai $ha^{-1}$) plus trifluralin (1,400 g ai $ha^{-1}$) were applied preplant incorporated. Detailed information of herbicides utilized can be found in Table 1.

The experimental design was a randomized complete block with an incomplete factorial arrangement of treatments. Treatments included the dimethylamine salt of 2,4-D (0, 1,120, and 2,240 g $ha^{-1}$) and soybean growth stage (V5, R2, or V5 followed by R2). Treatments were replicated four times. The expected 2,4-D field use rates for 2,4-D-resistant soybean ranged from 560



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Table 1. Sources of material for herbicides applied to experiments.

| Common name | Trade name | Active ingredient | Manufacturer |
|---|---|---|---|
| Imazethapyr | Pursuit | 240 g $L^{-1}$ | BASF Corporation Agricultural Products, Research Triangle Park, NC 27709, www.agro.basf.com |
| Metribuzin plus Chlorimuron-ethyl | Canopy DF | 0.643 g $g^{-1}$<br>0.107 g $g^{-1}$ | DuPont Crop Protection, Wilmington, DE 19898, www.dupont.com |
| Trifluralin | Treflan HFP | 480 g $L^{-1}$ | Dow AgroSciences, Indianapolis, IN 46268, www.dowagro.com |
| Sulfentrazone plus Cloransulam-methyl | Sonic | 0.62 g $g^{-1}$<br>0.08 g $g^{-1}$ | Dow AgroSciences, Indianapolis, IN 46268, www.dowagro.com |
| 2,4-D (dimethylamine salt) | Weedar 64 | 456 g $L^{-1}$ | Nufarm, Inc., Burr Ridge, IL 60527, www.nufarm.com/US |

to 1,120 g $ha^{-1}$ 2,4-D per application. The 2,240 g $ha^{-1}$ rate of 2,4-D was included to determine the extent of crop safety enabled by AAD-12, whereas sequential treatments were used to simulate two postemergence treatments. In 2008, sequential treatments were not applied. All 2,4-D treatments were applied in a water carrier volume of 140 L $ha^{-1}$ at 138 kPa using a $CO_2$-pressurized backpack sprayer with a 2.3 m wide boom equipped with XR11002 flat-fan nozzles (TeeJet Spraying Systems Company, Wheaton, IL 60189).

Visible estimates of soybean injury from 0 (no injury) to 100% (complete plant death) were collected at 3, 7, and 14 d after treatment (DAT) at PPAC and MRC in 2009 and 2011. Crop injury data were not taken in 2008. At maturity (at all sites and years), 10 arbitrarily selected plants from the middle two rows (five consecutive plants from each row) of each treatment were selected to determine the following yield components as outlined by Board and Modali (2005): seed mass (grams per 100 seed), seeds $m^{-2}$, seeds $pod^{-1}$, pods $m^{-2}$, main stem reproductive nodes $m^{-2}$, pods reproductive $node^{-1}$, main stem nodes $m^{-2}$, and percent reproductive nodes. Remaining soybean from the plot was machine harvested and was adjusted to 130 g $kg^{-1}$ moisture.

Data were subjected to ANOVA using PROC MIXED in SAS (The SAS System for Windows, Version 9.2, SAS Institute Inc., Cary, NC 27513) to test for significant effects ($P \leq 0.05$) of 2,4-D rate, application timing, and 2,4-D rate by application timing. These parameters were treated as fixed effects. Location and year were treated as random variables because there was homogeneity of error variances. Tukey pairwise comparison was used for means separation.

Table 2. Estimated soybean visible injury (0 to 100%) at 3, 7, and 14 d after treatment (DAT). Studies were conducted at Dow AgroSciences Midwest Research Center (MRC) near Fowler, IN and Pinney Purdue Agriculture Center (PPAC) near Wanatah, IN in 2009 and 2011.

| 2,4-D rate | Treatment stage | Visible injury | | |
|---|---|---|---|---|
| | | 3 DAT | 7 DAT | 14 DAT |
| g ae $ha^{-1}$ | | (%) | | |
| 0 | Untreated check | 0 c[a] | 0 b | 0 b |
| 1,120 | V5 | 0 c | 0 b | 1 b |
| 1,120 | R2 | 0 c | 1 b | 0 b |
| 1,120 fb[b] 1,120 | **V5**[c] fb R2 | 0 c | 0 b | 0 b |
| 1,120 fb 1,120 | V5 fb **R2** | 0 c | 0 b | 0 b |
| 2,240 | V5 | 1 bc | 1 b | 1 b |
| 2,240 | R2 | 2 a | 2 a | 1 ab |
| 2,240 fb 2,240 | **V5** fb R2 | 2 ab | 1 b | 1 b |
| 2,240 fb 2,240 | V5 fb **R2** | 2 a | 2 a | 3 a |

[a] Means within a column followed by the same letter are not significantly different according to Tukey's pairwise comparison ($P \leq 0.05$).

[b] Abbreviation: fb, followed by.

[c] Bold typeface indicates the growth stage when the assessment was taken in the sequential series (V5 fb R2 soybean growth stage).

## Results and Discussion

The sensitivity of AAD-12 soybean to 2,4-D applied at the V5 and R2 growth stages was minor. Regardless of rate, 2,4-D applied to AAD-12 soybean caused ≤ 3% soybean injury at all evaluation dates (Table 2; Figure 1A–1C), which is far below industry standards of 10%. Treatments of 1,120 g $ha^{-1}$ 2,4-D caused up to 0, 1, and 1% soybean injury, respectively, at 3, 7, and 14 DAT. Treatments of 2,240 g $ha^{-1}$ 2,4-D caused up to 2, 2, and 3% soybean injury, respectively, at 3, 7, and 14 DAT. Injury symptoms were not epinastic, which are often associated with 2,4-D injury (Figure 1D–1E). Rather, injury was typically small necrotic spots on the leaves, which is atypical of 2,4-D injury to glyphosate-sensitive soybean. The necrotic spots remained on the leaves, but were soon covered by new leaf growth. Injury might have been caused by a high formulation of active 2,4-D



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Figure 1. Aryloxyalkanoate dioxygenase-12 soybean plants treated with (A) 0, (B) 1,120, and (C) 2,240 g $ha^{-1}$ 2,4-D, 3 d after treatment (DAT) at the R2 soybean growth stage. (D and E) 2,4-D-sensitive soybean treated with 11.2 g $ha^{-1}$ 2,4-D, 14 DAT at the V5 soybean growth stage, and (F) 2,4-D-sensitive soybean treated with 2,240 g $ha^{-1}$ 2,4-D, 8 DAT at the R2 soybean growth stage. Images D, E, and F from Robinson et al. (2013a).

to a localized region on the plant leaf, causing cell leakage and eventual cell death, or inert ingredients in the herbicide formulation might have caused the damage. A similar response was observed at 8 DAT when Robinson et al. (2013a) applied a high concentration of 2,4-D (2,240 g $ha^{-1}$) to 2,4-D-sensitive soybean (Figure 1F). However, 2,4-D was apparently metabolized by AAD-12 soybean in this research, resulting in no further injury, whereas 2,4-D applied to 2,4-D-sensitive soybean resulted in plant death.

AAD-12 soybean seed yield components were not affected by 2,4-D application rate, application timing, or 2,4-D rate by application timing (Table 3). Thus, no effect of 2,4-D was observed on AAD-12 soybean yield when 2,4-D treatments were applied before flowering, during flowering, or before and during flowering. Average yields from

Table 3. P values from the effect of 2,4-D rate[a] and application timing[b] on soybean yield components at Dow AgroSciences Midwest Research Center (MRC) near Fowler, IN in 2008, 2009, and 2011 and Pinney Purdue Agriculture Center (PPAC) near Wanatah, IN in 2009 and 2011.

| Yield component | 2,4-D rate | Treatment timing | 2,4-D rate by treatment timing |
|---|---|---|---|
| | | P value | |
| Seed yield | 0.3896 | 0.4826 | 0.8361 |
| Seed number | 0.1832 | 0.2796 | 0.7312 |
| Seed mass | 0.2846 | 0.5477 | 0.8143 |
| Seed per pod | 0.1491 | 0.1366 | 0.6204 |
| Pod number | 0.0634 | 0.1467 | 0.5248 |
| Pod per reproductive node | 0.3652 | 0.4733 | 0.1070 |
| Reproductive node number | 0.1128 | 0.3889 | 0.1376 |
| Percentage reproductive nodes | 0.4894 | 0.7356 | 0.2400 |
| Node number | 0.2093 | 0.5190 | 0.1412 |

[a] 2,4-D rates: 0, 1,120, and 2,240 g $ha^{-1}$.
[b] Treatment timings: V5 and R2 in 2008 and V5, R2, and V5 followed by R2 soybean growth stages in 2009 and 2011.

untreated plots ranged from 2.9 to 5.1 Mg $ha^{-1}$ when adjusted to 13% moisture. In other research an 11% yield loss was reported when 70 and 140 g $ha^{-1}$ 2,4-D treatments were applied to 2,4-D-sensitive soybean at the soybean bloom stage (Wax et al. 1969) and a yield reduction of 32% was reported when 112 g $ha^{-1}$ 2,4-D was applied on V3 to 2,4-D-sensitive soybean (Andersen et al. 2004). At 560 g $ha^{-1}$ 2,4-D, Slife (1956) found a 21 to 62% yield loss in 2,4-D-sensitive soybean. In another study that examined yield components of 2,4-D sensitive soybean, 2,4-D at 87 to 116 g $ha^{-1}$ reduced yield by 5%; however, rates of 115 to 389 g $ha^{-1}$ were required to reduce main stem nodes, reproductive nodes, pod number, and seed by 5% (Robinson et al. 2013a).

We found that minimal soybean injury occurred after 2,4-D treatments were applied and did not reduce the yield or yield components of AAD-12 soybean. Other researchers reported that glyphosate treatment to glyphosate-resistant soybean caused up to 10% soybean injury, and yet there was no reduction in yield (Krausz and Young 2001). The AAD12 technology will allow the use of an alternative herbicide mode of action for postemergence weed control applications in soybean, which might be especially advantageous for the control of glyphosate-resistant weeds. However, this technology needs to be utilized with prudence to avoid development of weed resistance to 2,4-D.

In our study we evaluated AAD-12 soybean at two locations in Indiana without severe drought or temperature conditions. As was found in glyphosate-resistant soybean, adverse climate conditions caused soybean to respond in an unexpected manner (King et al. 2001; Zobiole et al. 2012). In our study, the climate conditions did not result in soybean stress. Therefore, further research should evaluate 2,4-D applications on AAD-12 soybean under drought and temperature stress to ensure that AAD-12 soybean growth and production is not affected.

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