# UPTAKE, TRANSLOCATION, AND METABOLISM 2,4-D IN ENLIST CROPS AND CONTROL OF DROUGHT-STRESSED WATERHEMP (AMARANTHUS TUBERCULATUS) WITH 2,4-D AND GLYPHOSATE

BY

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DISSERTATION

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**ABSTRACT**

The synthetic auxin herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), is one of the oldest and most widely-used herbicides in the world and is a systemic, postemergence (POST) herbicide that is selective in monocots such as corn, wheat, sorghum, and turf, but dicots are sensitive to 2,4-D. Auxin herbicides mimic and induce similar responses as the natural phytohormone, indole-3-acetic acid. Sensitive dicots are controlled through a cascade of events derived from elevated cellular levels of 2,4-D, resulting in increased phytohormone levels, reactive oxygen species, cell membrane injury, and ultimately plant death. Selectivity of 2,4-D is derived from metabolism differences between monocots and dicots.

Until recently, 2,4-D could not be used in dicot crops like soybean (*Glycine max*) and cotton (*Gossypium hirsutum*); however, genetically-modified varieties are being developed. The Enlist Weed Control System™ developed by Dow AgroSciences will confer 2,4-D resistance in several crops including corn (*Zea mays*), soybean, and cotton. Resistance is derived from insertion of a transgene from the soil bacteria enzyme family, aryloxyalkanoate dioxygenase (AAD), which can metabolize 2,4-D to the nonherbicidal metabolite, dichlorophenol.

During the development of Enlist soybean, injury was observed to treated leaves following certain herbicide applications, which is atypical of synthetic auxins. When the premixed product, Enlist Duo (2,4-D choline, glyphosate, and specific adjuvant package (Adj.)) or the tank-mixture of 2,4-D choline + glyphosate + Adj. was applied to Enlist soybean, small necrotic spots formed on treated leaves, but injury to Enlist corn was less frequent. Injury to Enlist soybean was unexpected due to the previously determined rapid rate of 2,4-D metabolism by the AAD enzyme. To develop a better understanding of how and why this injury occurs in Enlist soybean, research was conducted to measure uptake, translocation, and metabolism of 2,4-D in Enlist soybean, Enlist corn, and non-transformed varieties utilizing radiolabeled 2,4-D in a whole-plant and an excised-leaf assay. It was concluded that

enhanced 2,4-D uptake with the Enlist Duo treatment leads to injury in Enlist soybean. In both Enlist crop varieties, the rate and/or amount of 2,4-D metabolism were greater relative to non-transformed varieties and glyphosate did not affect 2,4-D metabolism. In the Enlist Duo treatment, 2,4-D uptake is very rapid and greater than with other treatments in soybean. In corn, 2,4-D uptake levels were much lower than detected in soybean, and surprisingly Enlist Duo resulted in the least amount of uptake. When the concentration of free 2,4-D was reduced in soybean by utilizing the ester formulation of 2,4-D, injury to Enlist soybean was eliminated. By tank mixing a chloroacetamide herbicide with Enlist Duo, injury was observed in Enlist corn and 2,4-D uptake was increased compared to using Enlist Duo alone.

Excessive 2,4-D uptake levels derived from Enlist Duo result in injury to Enlist soybean. The rapid influx of 2,4-D into soybean may overwhelm or exceed the metabolic capacity of the AAD enzyme, resulting in a pool of free 2,4-D acid in the soybean plant. Injury is reduced when 2,4-D ester is used instead of 2,4-D choline because 2,4-D ester must be converted to the active form of 2,4-D acid by esterases located within the cuticle and/or apoplast, limiting the influx and amount of 2,4-D that must be metabolized by AAD. Reducing 2,4-D uptake in Enlist crops by altering the adjuvants or changing the formulation of 2,4-D would reduce the risk of crop injury, but 2,4-D uptake in weeds and efficacy may be compromised.

Weed control with POST herbicides can be affected by many factors including the growing conditions during the application. An environmental condition known to alter POST herbicide efficacy is drought. Plants under drought stress (water stress) tend to be more difficult to control compared to unstressed plants. With predicted climate changes, a greater potential for periods of less rain or more frequent droughts may drive the need to maintain weed control levels when plants are drought stressed. The objectives of this research were to determine the effect of drought stress on waterhemp (*Amaranthus tuberculatus*) control POST with 2,4-D and/or glyphosate, and to define potential differences between the two herbicides in relation to drought stress and POST activity.

Greenhouse assays and a whole-plant assay utilizing radiolabeled herbicides were conducted to investigate levels of waterhemp control in relation to varying drought stress levels, timing of the stress, and their effect on herbicide uptake and translocation. Levels of waterhemp control were determined in the greenhouse with varying rates of 2,4-D and glyphosate (less than labeled rates; termed “low” and “high”), as well as tank mixtures of these two herbicides, under varying levels of water stress created by watering the plants with 10, 20, or 40 mL of water per day. Another greenhouse assay was utilized to determine waterhemp control with the two herbicides under different timings of water stress. The drought condition occurred either one week before the herbicide application, one week after the herbicide application, or during the full two-week period.

Herbicide efficacy increased as the amount of water supplied per day increased. At high-stress levels, the reduction of waterhemp dry matter was greater with 2,4-D-low compared to glyphosate-low and was equivalent to glyphosate-high. Herbicide efficacy was greatest when the drought stress occurred before the herbicide application and when plants were watered to saturation after the application. When the drought stress occurred immediately after the herbicide application, waterhemp dry matter levels were equal to plants held under drought stress both before and after the herbicide application. Uptake and translocation of radiolabeled glyphosate was significantly less in plants under drought stress, while uptake and translocation of radiolabeled 2,4-D was not altered.

Significant dry matter reduction in waterhemp plants under drought stress is possible with 2,4-D compared to glyphosate at the rates examined in the greenhouse study. Greater uptake and translocation of 2,4-D in drought-stressed waterhemp plants may have contributed the greater herbicide efficacy achieved with 2,4-D compared to glyphosate. With both herbicides, higher efficacy levels were attained when plants were not stressed after the herbicide application. Whenever possible, timing POST herbicide applications in accordance with rainfall events and selecting the appropriate

herbicide-adjuvant combination can increase waterhemp control, even if the plants are stressed prior to the application.

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# CHAPTER 1

## LITERATURE REVIEW AND ATTRIBUTIONS

### History and Use of 2,4-D

The herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), was discovered in 1941 and developed as a potential chemical warfare agent (Cobb and Reade 2010). Though it had not yet been used as a plant growth regulator, Zimmerman and Hitchcock characterized the natural auxin-like properties of 2,4-D that led to its development as a commercial herbicide (Cobb and Reade 2010). The first members of the phenoxyacetic acid family of the auxin-regulating herbicides were 2,4-D and 2-methyl-4-chlorophenoxyacetic acid (MCPA), and 2,4-D was marketed as a commercial herbicide in 1945 as "Weedone" making 2,4-D one of the oldest herbicides in agriculture (Cobb and Reade 2010). The phenoxyacetic acid family became a success and was influential on agriculture because these herbicides provided selective control of broadleaf weeds in cereal crops, were non-toxic, effective at low doses, and inexpensive to produce (Cobb and Reade 2010). The success of 2,4-D and MCPA increased the investment and research of new herbicides establishing the importance of chemical weed control in modern agriculture (Cobb and Reade 2010). Currently, the phenoxy herbicides are one of the most widely-used families of selective herbicides on a global scale and play a major role in dicot weed management (Cobb and Reade 2010).

A systemic (phloem and xylem mobile), selective, and postemergence (POST) herbicide, 2,4-D has a wide range of uses. 2,4-D is used in agricultural crops such as corn (*Zea mays*), wheat (*Triticum* spp.), and rice (*Oryza sativa*), but also non-agricultural crops like turf. As an auxin-regulating herbicide, 2,4-D mimics the natural plant hormone, indole-3-acetic acid (IAA) (Cobb and Reade 2010; Zazimalova et al. 2014). IAA plays a major role in several plant physiological responses including morphology,

geotropism, phototropism, apical dominance, leaf senescence, abscission, flowering, and fruiting due to its key role in cell division, elongation, and differentiation (Cobb and Reade 2010; Zazimalova et al. 2014). Exogenous levels of auxins can stimulate and inhibit plant growth in a concentration-dependent manner, allowing auxin mimics such as 2,4-D to be used as plant growth regulators (Cobb and Reade 2010; Fedtke and Duke 2005; Zazimalova et al. 2014). Natural auxin concentrations are tightly regulated in the plant by biosynthesis, degradation, and conjugation in order to produce the desired physiological response (Cobb and Reade 2010; Fedtke and Duke 2005; Zazimalova et al. 2014). Only the active IAA acid form produces a response. The active IAA pool within the plant can be increased by synthesizing more IAA and reduced by oxidative degradation via an IAA oxidase that permanently inactivates IAA or conjugating materials to IAA (Cobb and Reade 2010; Fedtke and Duke 2005; Zazimalova et al. 2014). Conjugation of IAA with glucose, amino acids, and myo-inositol temporarily inactivates or reduces potency of IAA and can be hydrolyzed back to the active form when needed (Cobb and Reade 2010; Fedtke and Duke 2005; Zazimalova et al. 2014). These two varying methods of inactivating IAA directly relates to selectivity of 2,4-D in crops (Fedtke and Duke 2005; Pillmoor and Guant 1981; Sterling and Hall 1997) and will be discussed in more detail in a later section.

The mode of action of 2,4-D and other auxin herbicides is very complex, and advances have recently been made at a molecular level but only in *Arabidopsis* (Enders and Strader 2015; Grossman 2010). Previously, researchers believed auxin herbicides were able to control weeds through uncontrollable growth. Many researchers believed that the massive cell division generated by auxin herbicides resulted in phloem collapse and therefore “strangulation” of the weed (Pillmoor and Gaunt 1981). Further research into the biochemical effects of auxin herbicides led to the idea of a cascade of reactions that lead to plant death (Sterling and Hall 1997). Recent discoveries and a better understanding of auxin perception and signaling in plants (Enders and Strader 2015; Grones and Friml

2015) has allowed for proposing more complete models to describe the mode of action of auxin herbicides. There are several key auxin receptors in plants including Transport Inhibitor Resistant 1 (TIR1) and several homologous proteins in *Arabidopsis* called Auxin signaling F-Box (AFBs) that localize to the nucleus, and until recently, the plasmalemma-localized Auxin-Binding Protein 1 (ABP1) (Grones and Friml 2015; Grossman 2010; Zazimalova et al. 2014). These proteins are the most understood, but there are many other proteins that bind auxins indicating there may be other receptors (Zazimalova et al. 2014), and recently it was discovered ABP1 is not required for auxin signaling, auxin-responsive gene expression, or regulation of *Arabidopsis* development (Gao et al. 2015). Auxin binding to these receptor proteins allows for auxin-related gene expression to occur under low concentrations of IAA or an herbicide because auxin-regulated genes are repressed by Aux/IAA transcriptional repressor proteins (Grossman 2010). During situations when high IAA or auxin herbicide concentrations exist, these compounds act as 'molecular glue' by increasing the binding interaction between the TIR1/AFB receptor protein and Aux/IAA repressors (Grossman 2010; Zazimalova et al. 2014). The Aux/IAA transcriptional repressors are ubiquitin tagged and subsequently degraded, which releases auxin-related genes from repression by allowing Auxin Response Factors (ARFs) to bind to their target gene promoters (Grossman 2010; Zazimalova et al. 2014). Gene expression can be down-regulated by the natural metabolism of IAA, but with auxin herbicides in sensitive plants the auxin-responsive gene expression is not repressed. The lack of auxin herbicide detoxification and continuous gene expression in sensitive weeds results in a cascading effect of many biochemical and physiological events leading to plant death (Fedtke and Duke 2005; Grossman 201;). The cascading events can be divided into three distinct phases: (1) stimulation, (2) inhibition, and (3) decay (Fedtke and Duke 2005; Grossman 2010). The stimulation phase is characterized by a large increase in synthetic auxin concentration directly after the herbicide application resulting in the physical symptoms of stem curling and epinasty, but also the metabolic activation of ion channels, ATPases, enzyme synthesis, ethylene formation, and abscisic acid (ABA) accumulation

(Grossman 2010). The inhibition phase occurs a day after the herbicide application and consists of stunted growth, intensified leaf pigmentation, stomatal closure, reduced transpiration and carbon fixation, and the production of reactive oxygen species (ROS) (Grossman 2010). The decay phase occurs within three days after application and consists of cell and plant death characterized by destruction of chloroplasts, membranes, and vascular system (Grossman 2010).

This complex mode of action is effective at controlling sensitive dicot weeds but also may be a reason for the limited amount of auxin herbicide-resistant weeds. Currently there are 32 weed species resistant to auxin herbicides globally, and 28 dicots resistant to 2,4-D specifically, and some species are cross resistant to other auxin herbicides (Heap 2015). Auxin resistance is uncommon in the United States with only eight resistant dicot species and five 2,4-D resistant dicot species, with some species having resistance to other auxin herbicides (Heap 2015). Despite being used for almost 70 years, the number of resistant weeds is relatively low compared to other herbicide families, particularly the acetolactate synthase and photosystem II-inhibiting herbicides. The low amount of resistance may be attributed to the presence of rare or recessive alleles conferring resistance to auxin herbicides, the potential for fitness penalties due to mutations conferring resistance, the complex mode of action, and multiple potential target sites for auxin herbicides (Mithila et al. 2011). The potential for increased use of 2,4-D and dicamba in the future due to commercialization of new auxin herbicide-resistant crop species may result in selection of more resistant weed populations, however (Mortensen et al. 2012).

**Genetically Engineered Crops and 2,4-D**

Genetically-engineered crops that confer resistance to 2,4-D and other herbicides are currently being developed and released (Wright et al. 2010). With the increased need to control dicot weeds with multiple-herbicide resistance mechanisms (Ma et al. 2013), utilizing 2,4-D in crops that have engineered

resistance will provide growers with a new tool for selective weed control. The Enlist™ Weed Control System developed by Dow AgroSciences provides a new, novel means of 2,4-D resistance in several crops including soybean (*Glycine max*), cotton (*Gossypium hirsutum*), and corn. The Enlist™ Weed Control System will utilize a new formulation of 2,4-D acid, the choline salt, which displays many improvements compared to previous 2,4-D amine salt formulations including less potential for volatility and off-target movement as well as reduced odor. The new 2,4-D formulation will be used with glyphosate in the premix product Enlist™ Duo. The use of the Enlist™ Weed Control System will provide growers with a new option for weed control in both soybean and cotton since 2,4-D was previously not able to be used in these crops.

Engineered crop resistance to 2,4-D is derived from the insertion of homologs of the *TfdA* transgene that encode the soil bacteria enzyme class aryloxyalkanoate dioxygenase (AAD) (Wright et al. 2010). The AADs catalyze the cleavage of 2,4-D to two nonherbicidal metabolites, dichlorophenol (DCP) and glyoxylate (Wright et al. 2010). DCP is rapidly metabolized in sensitive and 2,4-D-resistant dicot species to various glucosides by naturally occurring glucosyltransferases (Gallandt and Balke 1995; Laurent et al. 2006; Schmitt et al. 1985). Two isoforms of the AAD enzyme will be used depending on the crop. In corn, the AAD-1 enzyme derived from the *RdpA* gene is used, and in soybean and cotton the AAD-12 enzyme derived from the *SdpA* gene is used (Wright et al. 2010). The two homologs are used because they provide different levels of resistance to 2,4-D and other herbicide classes (both auxin and non-auxin herbicides). For example, the AAD-1 enzyme provides less resistance to 2,4-D but provides resistance in corn to the graminicide herbicide family of acetyl-CoA-carboxylase inhibitors, the aryloxyphenoxy-propionates (AOPPs), rendering AAD-1 unique since the use of AOPP herbicides in corn was not previously possible (Wright et al. 2010). The AAD-12 enzyme was used in dicot crops because it provides a greater level of resistance to 2,4-D compared to AAD-1, and AAD-12 also provides resistance

to another auxin herbicide family, pyridyloxyacetate (Wright et al. 2010). The AAD enzymes will be stacked with other herbicide resistances in either a breeding or molecular stack that provides herbicide resistance to 2,4-D, as well as glyphosate and glufosinate (Wright et al. 2010).

**Uptake and Translocation of 2,4-D**

Many studies and reviews on 2,4-D uptake and translocation have been published, with the majority of the research conducted prior to 1980. However, in the period since then, the amount of research on 2,4-D uptake and translocation has declined. Previous reviews have discussed the results of early research (Currier and Dybing 1959; Franke 1967; Pillmoor and Gaunt 1981; Richardson 1977; Robertson and Kirkwood 1969, 1970; Sargent 1965), but more recent physiological research has not been reviewed.

The objectives of this section are to summarize the research on 2,4-D uptake and translocation. Many plant, environmental, and chemical factors can influence 2,4-D uptake, but translocation is less frequently altered. The myriad of factors influencing uptake will be discussed below, including any potential effect on translocation. Resistance to 2,4-D in certain weed species populations may also be related to impaired uptake and translocation, but these populations and resistance mechanisms will be discussed in a later section.

*Environmental factors*

***Light.*** Light intensity and quality can alter herbicide uptake and translocation by dictating photosynthesis, photoassimilate transport, and cuticle characteristics. Limited research has been conducted on 2,4-D uptake and translocation as affected by light. Uptake of 2,4-D was always increased in the presence of light compared to dark and was increased with varying rates and maximum values of uptake in six different plant species (Sargent and Blackman 1972). Light intensity did not affect 2,4-D

uptake in hemp dogbane (*Apocynum cannabinum*), but increasing light intensity to 75 klux from 50 klux resulted in 14% greater movement out of the treated leaf (Shultz and Burnside 1980).

***Temperature.*** Temperature can alter herbicide uptake and translocation by influencing photosynthetic rates, altering the cuticle, and many other plant-related processes. Higher temperatures, to a limit, result in greater photosynthesis and photoassimilate production, greater enzyme activity, and greater phloem loading, potentially increasing herbicide translocation. The physical state of the plant cuticle and stomatal openings can be altered through increased temperatures, which influence herbicide uptake (Kirkwood 1999). In general, 2,4-D uptake and translocation is greater at higher temperatures. Uptake of 2,4-D increased in aspen poplar (*Populus tremuloides*) (Sharma and Vanden Born 1970) and in red kidney beans (*Phaseolus vulgaris* L.) at higher temperatures (Pallas 1960). Translocation of 2,4-D in red kidney beans increased as air temperatures increased (Pallas 1960). Uptake in hemp dogbane increased at 30˚C compared to 25˚C, but translocation was not altered (Schultz and Burnside 1980).

***Humidity.*** The relative humidity during an herbicide application can influence herbicide uptake by influencing stomatal conductance, herbicide droplet drying time, and other factors, but alteration of translocation is not typical. Increased levels of humidity have been shown to result in greater 2,4-D uptake and translocation. Uptake of 2,4-D doubled under high humidity conditions in poplar, and when spray droplets were rewetted after drying under low humidity conditions, 2,4-D uptake was increased (Sharma and Vanden Born 1970). Uptake was greater at humidity levels ranging from 70-74% compared to 34-48%, and a difference between the drying times of herbicide spray droplets under the two humidity levels was not measured (Pallas 1960). Uptake was greater when the humidity was 100% compared to 40%, and the rate of translocation to the meristematic region and rhizomes was much faster at 100% relative humidity in wolftail (*Carex cherokeensis* Schwein) (Burns et al. 1969). When the

humidity increased from 40% to 90%, 2,4-D uptake was 11% greater in grapes (*Vitis vinifera* cv. Lemberger), but an effect on translocation was not measured (Al-Khatib et al. 1992).

***Water Stress.*** Herbicide efficacy can be altered by water or drought stress in several herbicides due to reduced uptake and translocation. Plants under water or drought stress might have reduced herbicide uptake levels due to reduced stomatal openings and thicker or altered cuticle. Translocation may be reduced because of less photosynthesis due to reduced water availability and gas exchange resulting in less photoassimilate production and transport. Drought stress typically does not affect 2,4-D uptake, but translocation levels are reduced. The uptake of 2,4-D acid was similar among turgidity levels ranging from 66% to 88% in bean (*Phaseolus vulgaris*), but translocation from the treated leaf decreased when the soil moisture was at 13% (Basler et al. 1961). Similarly, 2,4-D trimethylamine salt uptake among various soil moisture contents did not differ, but translocation was twice as great at 0.3-atm soil tension compared to 4-atm (Pallas and Williams 1962). In contrast to creating drought stress through the soil, stress in soybean (*Glycine max*) was created by utilizing polyethylene glycol solution (PEG) (Kogan and Bayer 1996). When PEG was used, plant relative water content was reduced from 88% to 74.6% and leaf water potential was reduced from -0.55 to -1.21 MPa resulting in 39.7% less 2,4-D foliar influx in soybean leaves under conditions that mimic water stress (Kogan and Bayer 1996). However, conditions were brief and did not include a preconditioning stage for the plants to develop or alter the leaf anatomy that would be observed in water-stressed plants (Kogan and Bayer 1996).

*Plant Factors*

***Growth stage and environment.*** The age of the plant, leaf, and growing conditions can all affect herbicide uptake and translocation. Leaf age can influence herbicide uptake through the development and maturity of the cuticle. The environment or growing conditions in which a plant develops can alter

herbicide uptake and translocation. Since translocation of herbicides occurs in a source to sink fashion, translocation out of young, developing leaves may differ from older, mature ones. The effect of leaf age and environment on 2,4-D uptake can vary depending on species. Greater uptake was found in immature leaves of bean, pea (*Pisum sativum)*, beet (*Beta vulgaris*), and sunflower (*Helianthus annus*) than mature leaves, but a difference in uptake was not measured among leaf ages in corn or cotton (Sargent and Blackman 1972). Developing leaves contain epicuticular wax that is less thick and has varying composition compared to mature leaves, which can influence herbicide uptake (Baker and Hunt 1981). Reduced uptake in five-wk-old field bindweed (*Convolvulus arvensis* L.) seedlings was measured compared to 6 and 16 wk old mature plants, but greater translocation occurred in seedlings (Agbakoba and Goodin 1969). Grapes grown in the field had 26% less uptake compared to grapes grown in the greenhouse and was attributed to cuticle differences (Al-Khatib et al. 1992).

***Leaf and cell factors.*** Several features and characteristics of plant leaves can influence uptake and translocation of herbicides. Leaf angle, cuticle, pubescence, and others can alter how much herbicide gets into the plant, and the structure of plant cells within the leaf and stem can affect herbicide translocation. Many studies have been conducted to determine the effect of cuticle thickness and composition on 2,4-D uptake in various species. Penetration of 2,4-D was not correlated to cuticle thickness as measured in different species with varying tolerance levels (Norris 1974). A study of cuticle composition and chemistry found a wide variability among several plant species ranging in sensitivity, but no relationship with 2,4-D uptake was found (Baker and Bukovac 1971). Even with varying leaf characteristics, no difference in 2,4-D uptake was found at 1, 3, or 7 d after application in alfalfa (*Medicago sativa*), grape, and pea (Al-Khatib et al. 1992). Uptake of 2,4-D correlates with the amount of stomatal openings (Pallas 1960), even though overall uptake through the stomata is small.

Translocation of 2,4-D follows the phloem-loading pathway and has been reviewed previously (Devine and Hall, 1990). The general movement of assimilates and phloem-mobile herbicides occurs from regions of carbohydrate synthesis (source) to regions of storage or utilization (sink) (Devine and Hall, 1990). A concentration gradient drives transport between the source and sink, resulting in the movement of water containing dissolved solutes and herbicides (Devine and Hall, 1990). Herbicides and solutes can be transported either apoplastically (space between cells) or symplastically (directly between cells) resulting in different translocation rates and distribution (Devine and Hall, 1990). A strong symplastic movement of 2,4-D was measured in soybean, and 2,4-D was found predominately in transit areas like the stem (Martin and Edgington 1981). The 2,4-D molecule is transported in the phloem due to ion trapping (Riederer 2005). Weak acids, like 2,4-D, are trapped in the phloem due to the different pH levels between the phloem and xylem/apoplast (Riederer 2005). The phloem is more basic (pH 8.5) relative to the apoplast (pH 5.5), which causes the deprotonation of the weak acid to the negative ion preventing it from crossing the cell membrane and trapping the ionic form in the phloem (Riederer 2005). Translocation of 2,4-D in this way is similar to other systemic herbicides, but the presence of specific auxin herbicide carriers differentiates 2,4-D movement in the plant from most herbicides. Inter- and intra-cellular IAA movement is predominately dictated by auxin influx and efflux carriers (Enders and Strader 2015; Grones and Friml 2015). Within the cell, IAA concentration can be regulated by transporting IAA into the vacuole by the tonoplast-bound protein Walls Are Thin 1 (WAT) (Grones and Friml 2015). Inter-cellular efflux carriers include members of the PIN-Formed (PIN) and ATP-Binding Cassette Subfamily B (ABCB) families, and influx carriers include members of the Auxin Resistant 1/Like Aux1 (AUX1/LAX) family (Enders and Strader 2015). AUX1/LAX are plasma membrane bound only, while ABCB are plasma membrane and endomembrane localized and PIN carriers are found in the plasma membrane and endoplasmic reticulum (Enders and Strader 2015). The auxin influx carriers, AUX1, LAX1, and LAX3, transport both IAA and 2,4-D, and the efflux carriers, PIN2 and PIN7, can

transport 2,4-D while PIN1 cannot (Enders and Strader 2015). Fewer 2,4-D efflux carriers relative to influx carriers allow the concentration of 2,4-D to increase within the cell. In tobacco cells (*Nicotiana tabacum*), the concentration of IAA was 118 nM compared to 1106 nM 2,4-D concentration due to the lack of 2,4-D efflux from the cell (Delbarre et al. 1996). The concentration of 2,4-D in isolated potato (*Solanum tuberosum* cv. Yukon) tissue was 15-fold that of the ambient solution, and efflux of 2,4-D out of the treated tissue was very slow (Martin and Edgington 1981). Movement of 2,4-D out of the cell was not detected and viewed as unlikely due to the low affinity of 2,4-D to the auxin efflux carrier and slow diffusion rates (Delbarre et al. 1996). Increasing the concentration of 2,4-D acid within the cell allows greater herbicidal activity.

***Plant species effects.*** The uptake and translocation of 2,4-D are influenced by characteristics of each individual species. Many studies have measured the link between tolerance and sensitivity to 2,4-D uptake and/or translocation, in general, there is little correlation between sensitivity and uptake but a greater correlation with translocation (Pillmoor and Gaunt 1981).

The uptake of 2,4-D and plant sensitivity varies and results do not always correlate. The rate of uptake was slower in sensitive field bean than wild oat (Holloway and Edgerton 1992). By contrast, tolerant oat (*Avena sativa*) had faster uptake than sensitive soybean, but levels were equal by 24 h after application (Hall et al. 1982). Similar uptake levels were measured between tomato (*Solanum lycopersicum*) and eastern black nightshade (*Solanum ptychantum*), even though eastern black nightshade showed greater tolerance (Hall and Swanton 1988). However, greater uptake of two different 2,4-D formulations was measured in sensitive pea than tolerant eastern black nightshade (de Ruiter et al. 1993).

Though the link between uptake and tolerance is unclear, tolerant species tend to translocate less 2,4-D or differently compared to tolerant species (Pillmoor and Gaunt 1981). After 24 h, only 5% of total radioactivity moved from the treated leaf in tolerant oats compared to 55% in sensitive soybean, and more $^{14}$C-material was found in the roots (14.5%) and growing points (22.7%) in soybean compared to oats (3.1% and 1.6%) (Hall et al. 1982). Metabolism differences between tolerant and sensitive species (discussed in later section) could be an explanation for the measured differences in translocation. Rapid conversion of 2,4-D to metabolites that can be sequestered in the cells of tolerant species prevents the translocation of the active herbicide or metabolites compared to sensitive species.

Another means of tolerance for some species is via root exudation. Research on jimsonweed (*Datuva stramonium*), honeyvine milkweed (*Ampelamus albidus* (Nutt.) Britt.), and Canada thistle (*Cirsium arvense*) have suggested these species are more tolerant to 2,4-D by excreting the herbicide into the soil (Coble and Slife 1971; Fites et al. 1964; Turnbull and Stephenson 1985). Similarly, eastern black nightshade exuded 28% of applied 2,4-D into the soil compared to only 7% in tomato, and the extracted 2,4-D from the soil was unaltered (Hall and Swanton 1988).

*Chemical and application factors*

***pH.*** The pH of the spray solution can modify herbicide uptake and translocation. Altering the pH can dictate the ionic state of the herbicide molecule, and movement of a charged molecule across the cuticle and membranes is more difficult than an uncharged molecule. The pH of the spray solution has a more significant impact on uptake in comparison to translocation since the ionic state of the molecule will be dictated by the interior of the plant. Greater 2,4-D uptake at pH 3 than pH 5 was measured in bean and sunflower, and the change in pH had a more dramatic effect on uptake in sunflower (Szabo and Buchholtz 1961). Absorption of 2,4-D was greater at pH 3.5 compared to pH 8.5, and translocation

(as the percent absorbed by the plant) to the roots was greater at the lower pH in skeleton weed (*Chondrilla juncea* L.) (Greenham 1968).

***Effect of adjuvants.*** Adjuvants improve herbicide uptake by increasing leaf wetting, reducing herbicide droplet surface tension, improving leaf surface and droplet contact, and many others. In relation to 2,4-D, adjuvants improve uptake but the measured effect on translocation is not as significant. Depending on the formulation of 2,4-D, adjuvants can either increase or decrease uptake. Adjuvants increased the uptake of several 2,4-D formulations (amine, sodium salt, and isopropyl ester) in soybean and corn (Hauser 1955). However, adding the surfactant, Armoblen 600, increased uptake of the triethanolamine salt of 2,4-D but reduced uptake of the isooctyl ester formulation (de Ruiter et al. 1993). The uptake of 2,4-D acid in honeyvine milkweed increased 7 to 8-fold when 1.0% Tween 80 was added, and 2,4-D translocation increased with the adjuvant but the change was not as large (Coble et al. 1970). Similarly, uptake of 2,4-D triethanolamine salt was increased 4.8-fold in eastern black nightshade and 1.7-fold in pea when an adjuvant was included (de Ruiter et al. 1993). Translocation was not altered when an adjuvant was included with 2,4-D isooctyl ester or 2,4-D triethanolamine salt (de Ruiter et al. 1993).

Research has also been conducted on what specific adjuvant or what qualities in an adjuvant influence 2,4-D uptake. Uptake was 19% greater with the use of a mixture of organosilicone and acetylinic diol ethoxylate (ADE) surfactants compared to crop-oil concentrate in leafy spurge (*Euphorbia esula*) (Thompson et al. 1996). Ethylene oxide (EO) content was inversely related to 2,4-D uptake (Thompson et al. 1996), and surfactants with EO of 5 had greater uptake (85%) in broad bean (*Vicia faba* L.) compared to EO 10 (61%) and EO 14 (50%) (Liu 2004). A lower EO value corresponds to a shorter EO chain and less polar solution. Uptake was increased with a $C_{13}/C_{15}$ alkanol surfactant compared to a $C_{10}$ alkanol, and uptake was minimally increased using an octylphenol adjuvant (Liu 2004). However, 2,4-D uptake was not changed by surfactants with an EO value ranging from 6—18 in wild oat and bean in

another study (Holloway and Edgerton 1992). Specific adjuvant characteristics, physiochemical properties, and plant-leaf properties make it difficult to determine the general effect of adjuvants on 2,4-D uptake.

***Formulation and application methods of 2,4-D.*** The formulation of an herbicide can influence herbicide uptake and translocation. There are several formulations of 2,4-D that have been discussed in this review. Generally, uptake of 2,4-D as an ester is greater than an amine. In big leaf maple (*Acer macrophyllum* Pursh), uptake was greatest with the ethylhexyl ester formulation compared to the triethanolamine formulation (Norris and Freed 1966). Uptake of 2,4-D isooctyl ester was 2.4-times and 1.3-times greater in eastern black nightshade and pea, respectively, than the triethanolamine salt (2,4-D plus 2-hydroxythethyl amine), but after 24 h, there was no difference in translocation among the formulations (de Ruiter et al. 1993). Uptake of 2,4-D was greater with long chain amines like tetracyclamine and dodecylamine compared to short chain amines like dimethylamine salt in sunflower (Que Hee and Sutherland 1973).

The goal of an herbicide application is to deliver the active ingredient to the target weed. This can be influenced by several factors including the size of the spray droplets and carrier volume. These factors influence herbicide uptake by determining the spray coverage of the target, altering the concentration of the active ingredient in the droplet, and influencing droplet impact and retention on the leaf surface (Knoche 1994). Reducing the droplet size increases 2,4-D efficacy in several weeds, which was also documented with other auxin herbicides (dicamba and MCPA) but droplet size did not change glyphosate efficacy (Knoche 1994). Decreasing carrier volume did not decrease herbicide performance with 2,4-D, which is a similar response determined with many systemic herbicides; the exception is glyphosate, which exhibited greater efficacy (Knoche 1994). An optimum droplet size, carrier volume, or active ingredient concentration to maximize 2,4-D uptake in fava bean was not

determined, and increasing the amount of 2,4-D applied reduced the efficiency of uptake (Stevens and Bukovac 1987). Similarly, 2,4-D uptake as dimethylamine was not influenced by droplet size, but translocation decreased as droplet size increased in oriental mustard (*Sisymbrium orientale*) (Wolf et al. 1992). However, 2,4-D acid uptake was shown to be greatest with smaller droplets (0.5 µL compared to 10 µL) and with larger total volume (100 µL compared to 10 µL) applied to the leaf of *Phaseolus vulgaris* (Knoche and Bukovac 1999). The concentration of 2,4-D in droplets did not affect uptake, but translocation was reduced by 10% to 14% when the concentration increased 8-fold (Wolf et al. 1992). The true impact of droplet size and carrier volume on uptake and translocation may be difficult to determine due to experimental techniques and differences between target plants.

*Summary of uptake and translocation*

The uptake and translocation of 2,4-D is influenced by many factors but only a few have shown to be consistent. Uptake is greater under conditions of higher temperatures and humidity. Drought stress does not affect uptake, but translocation is reduced. Adjuvants, lower pH, and ester formulations of 2,4-D have greater uptake, but translocation is not altered. Uptake of 2,4-D has little correlation to cuticle thickness or composition and plant sensitivity, but sensitive species translocate more 2,4-D than tolerant species.

**Metabolism of 2,4-D in Plants**

Research on the metabolism of 2,4-D has been extensively studied since the 1950s. Recent reviews and information on 2,4-D and herbicide metabolism (Cobb and Reade 2010; Hatzios 2005) and many older reviews (Loos 1969; Pillmoor and Gaunt 1981; Roberston and Kirkwood 1970; Sandermann et al. 1984) provide in-depth information on 2,4-D metabolic pathways and enzyme reactions involved. Herbicide selectivity in many cases is dependent on plant metabolism. Plants metabolize herbicides by

converting the parent molecule to more polar products and insoluble residues (Hatzios 2005). Metabolism of 2,4-D occurs in sensitive and tolerant species and can occasionally be misleading because sensitive species may metabolize 2,4-D faster than tolerant species. Selectivity to 2,4-D is derived from detoxifying the herbicide in a permanent fashion compared to temporary conjugation mechanisms. Metabolites formed during 2,4-D metabolism have been measured in both sensitive dicots and tolerant monocots but vary in the proportion of each metabolite produced resulting in lower 2,4-D concentrations in tolerant monocots compared to dicots (Pillmoor and Gaunt 1981). The metabolism of 2,4-D is very similar to the natural metabolism of IAA. For example 2,4-D and IAA are both metabolized to temporary, reversible metabolites in dicots by modifying the carboxylic acid group, which can be converted back to active forms (Davidonis et al. 1980) by de-esterification. However, the selectivity of 2,4-D in monocots is primarily related to the formation and sequestration of irreversible, non-toxic metabolites, typically by modifications of the phenyl or heterocyclic ring (Feung et al. 1975).

The objectives of this section are to summarize 2,4-D metabolism by highlighting the various pathways, enzymes involved, metabolites formed, and differences among species. Metabolic detoxification of 2,4-D may be another mechanism for 2,4-D resistance in certain dicot weed populations, but these populations and possible resistance mechanisms will be discussed later.

*Side-chain cleavage*

Cleavage of the side-chain of 2,4-D has been measured in many plants, but only in a few species does metabolism play a major role, including red currant (*Ribes sativum* Syme), particular apple varieties (*Malus domestica*), strawberry (*Fragaria* x *ananassa*), and garden lilac (*Syringa vulgaris*) (Loos 1969). Side-chain degradation occurs through a single oxidation to yield glycolic acid and 2,4-dichlorophenol (Pillmoor and Gaunt 1981). A result of side-chain degradation and a means of measuring metabolism by

this pathway is a loss of radiolabeled carbon dioxide. Researchers monitoring 2,4-D metabolism by side-chain degradation are able to measure the loss of radiolabeled carbon dioxide. A range of 7 to 33% was determined in species that utilize this pathway compared to less than 1 to 2% in corn, soybean, and cotton with several other species producing less than 1% after 20 to 24 h, indicating how little this pathway is used in most plant species (Loos 1969).

*Direct conjugation*

Direct conjugation of 2,4-D with amino acids and glucose is another metabolic reaction in plants. Amino acids, mainly glutamate and aspartate, and glucose can be directly conjugated to the carboxylic acid group of 2,4-D to form amino acid conjugates or glucose esters. The formation of amino acid conjugates predominates in soybean (Sandermann et al. 1984) and other sensitive dicots (Hatzios 2005). Direct conjugation of IAA with amino acids occurs with the *GH3* gene, but this same enzyme cannot conjugate 2,4-D as a substrate (Staswick et al. 2005). The concentration of amino acid conjugates is much greater in comparison to other metabolites formed by dicots and the amount found in monocots. Amino acid conjugates are the first metabolite to form in dicots with glutamate conjugation appearing initially, but over time the 2,4-D-glutamate conjugate is converted to other metabolites, including the 2,4-D-aspartate conjugate and various sugar conjugates (Pillmoor and Gaunt 1981). Direct glucose conjugation of 2,4-D occurs with glucosyltransferase (GT) enzymes to form glucose esters (Hatzios 2005), and only glucose is used as the form of sugar (Pillmoor and Gaunt 1981). Though glucose is the only form of sugar used in direct conjugation, other sugars are utilized to form larger macromolecules in ring hydroxylation discussed later in this section. Generally, amino acid or glucose ester conjugates are more prevalent in sensitive dicots (Davidonis et al. 1982; Hatzios 2005), induce auxin-related activity similar to 2,4-D (Feung et al. 1974), and are readily hydrolyzed back to 2,4-D (Pillmoor and Gaunt 1981). This pool of active 2,4-D acid and reversible 2,4-D-conjugates allows the herbicide to exert its effects on

these species. However, 2,4-D conjugates have been recovered in the vacuoles of dicots (Sanderman et al, 1984) potentially reducing herbicidal activity. For further information on amino acid and glucose conjugate formation, several studies provide extensive detail about these metabolites (Feung et al 1973, 1975, 1978; Hamilton et al. 1971; Montgomery et al. 1971).

*Ring hydroxylation*

Tolerant monocots metabolize 2,4-D predominately through a ring hydroxylation reaction. A hydroxylation at the carbon-4 position on the aromatic ring of 2,4-D results in a migration or shift of the chlorine atom to the carbon-3 or carbon-5 position (Cobb and Reade 2010; Loos 1969; Pillmoor and Guant 1981). Ring hydroxylation occurs through a reaction catalyzed by cytochrome P450 (P450) enzymes (Hatzios 2005). The P450 enzyme family is involved in the metabolism and detoxification of several herbicides. The main metabolites formed from ring hydroxylation are 4-hydroxy-2,5-dichlorophenoxyacetic acid and 4-hydroxy-2,3-dicholorophenoxyacetic acid, and these metabolites are more readily observed in monocots than dicots (Sandermann et al. 1984). Other hydroxylated metabolites have been identified, but 4-hydroxy-2,5-D is the most common (Feung et al. 1971, 1975, 1978; Hamilton et al. 1971; Montgomery et al. 1971). Formation of *O*-glucosides of the ring hydroxylated metabolites by GT enzymes occurs rapidly after hydroxylation (Hatzios 2005). After glycolysation, metabolites can be further conjugated with other sugars, including malonic acid, to form larger structures. Malonylation occurs with *O*-malonyltransferase (*O*-MAT) enzymes, may help to stabilize conjugates against cellular digestion, and signals for the removal of the conjugates into the vacuole or across the plasma membrane (Hatzios 2005). Products from the ring hydroxylation metabolic pathway are more hydrophilic, non-phytotoxic, and polar compared with 2,4-D and cannot be hydrolyzed back to 2,4-D (Cobb and Reade 2010). These non-phytotoxic, irreversible ring-hydroxylates are more readily subjected to Phase III transport, detoxification, and sequestration mechanisms in

various subcellular locations, including the vacuole, or are incorporated with structural polymers like lignin, pectin, and cellulose in cell walls (Van Eerd et al. 2003; Hatzios 2005; Sandermann et al. 1984). While sensitive dicots can form ring-hydroxylated metabolites, they are usually detected at much lower concentrations (Feung et al. 1978) indicating the greater utilization of other metabolic pathways.

*Metabolism of alternate formulations of 2,4-D*

The acid of 2,4-D can be formulated as many different carboxyl esters (2,4-D ester) or as another herbicide, 2,4-dichlorophenoxybutyric acid (2,4-DB). Both of these unique formulations of 2,4-D are proherbicides or inactive forms of 2,4-D that must be converted to the active 2,4-D acid once inside the plant. Once converted to the acid, metabolism and plant responses occur in the same manner as if the acid was originally applied to the plant.

Ester formulations of 2,4-D have been previously discussed and generally result in greater uptake compared to amine formulations. Removal of the carboxyl ester chain from 2,4-D ester results in bioactivation to 2,4-D acid. Hydrolytic bioactivation of 2,4-D ester occurs by carboxylesterase enzymes (CXE) (Gershater and Edwards 2007). The specific CXE involved in 2,4-D ester activation was recently discovered and termed *At*CXE12 (Gershater and Edwards 2007).

A special formulation of 2,4-D, 2,4-DB, can be safely used in legume crops such as soybean and alfalfa. Bioactivation of 2,4-DB occurs through one round of β-oxidation in sensitive broadleaf weeds that consists of removing two carbons from butyric acid side-chain, resulting in conversion to 2,4-D acid (Cobb and Reade 2010). β-oxidation is a common reaction in fatty acid degradation that occurs in the peroxisomes via multi-functional enzymes (Poirier et al. 2006). Dicot selectivity to 2,4-DB is derived from the ability or inability to perform β-oxidation. For example, tolerant legume species do not activate 2,4-DB through β-oxidation, resulting in less active 2,4-D acid compared to sensitive dicot

species that perform the bioactivation step. Once 2,4-DB is converted to 2,4-D acid in sensitive dicots, further metabolism and plant responses are the same as if 2,4-D was applied.

*Storage of metabolites and bound residue*

During studies performed with radiolabeled 2,4-D, there is often a percentage of radioactivity that is not extractable with a solvent (Pillmoor and Gaunt 1981). In both monocots and dicots, metabolites are found in various subcellular locations including the vacuole or incorporated into cell walls with structural polymers like lignin, pectin, and cellulose, while unaltered 2,4-D has been found incorporated in the cell wall (Van Eerd et al. 2003; Hatzios 2005; Sandermann et al. 1984). The formation of bound residue is more common in monocots and is probably linked to the formation of hydroxylated metabolites and Phase III detoxification mechanisms (Hatzios 2005). For example, the amount of ethanol-insoluble material found in several monocot species ranged from 9 to 39% of the absorbed radioactivity compared to only 2 to 5% in dicot species (Pillmoor and Gaunt 1981). Insoluble fractions have been identified as the parent herbicide and derivatives of the 4-hydroxy-metabolites covalently incorporated in structural polymers (Hatzios 2005; Sandermann et al. 1984). Plant metabolism studies found metabolites located in the vacuole, and only 4-hydroxy-metabolites and amino acid conjugates were identified but not free 2,4-D acid (Hatzios 2005; Sandermann et al. 1984). Free 2,4-D acid may be transported into the vacuole (similar to IAA) by the WAT1 protein (Grones and Friml 2015), but further research is needed to determine if this protein also transports 2,4-D.

*Summary of plant metabolism*

The metabolism of 2,4-D occurs primarily through direct conjugation and ring hydroxylation, and to a smaller extent, side-chain cleavage. Herbicide selectivity is based on which metabolic pathway is predominantly utilized by the plant species. The direct conjugation of 2,4-D with amino acids or glucose

results in temporary metabolites that can by hydrolyzed back to 2,4-D acid, and is a more common reaction in sensitive dicots than in monocots. Ring hydroxylation of 2,4-D leads to the formation of a non- or partially-phytotoxic central metabolite which is further metabolized through conjugation reactions by GT and *O*-MAT enzymes to permanent, non-toxic compounds, and this detoxification pathway is more common in tolerant monocots. In both dicots and monocots, metabolites and free 2,4-D are incorporated with structural polymers in cell wall fraction, but only metabolites are found in the vacuole (Hatzios 2005; Sandermann 1984).

**Resistance to 2,4-D in Weeds**

As mentioned previously, resistance to 2,4-D in dicot weeds is relatively uncommon, especially in the United States (Heap 2015). The mechanism of resistance in most of these resistant dicot populations has not been determined but could potentially arise from reduced 2,4-D uptake or translocation, greater metabolism, impaired cellular transport, and/or an altered target site(s). A 2,4-D resistant population of wild mustard (*Sinapsis arvensis* L.) with similar 2,4-D uptake, translocation, and metabolism levels as compared with a sensitive population has reduced binding to ABP1 by the herbicide (Mithila et al. 2011, 2012). The resistant population exhibits a fitness penalty characterized by leaves with serrated edges that are smaller in area than plants from the sensitive population resulting in lower biomass and seed production in the resistant population compared to the sensitive (Mithila et al. 2011). The resistant wild mustard population might have a different mechanism of resistance though, since it was recently found that ABP1 is not required for auxin signaling or regulation of *Arabidopsis* development (Gao et al. 2015). This indicates that the lack of 2,4-D binding to ABP1 may not be the true resistance mechanism in this population. A population of prickly lettuce (*Lactuca serriola* L.) resistant to 2,4-D displayed reduced uptake and translocation compared to the sensitive population, but rates of 2,4-D metabolism were the same (Riar et al. 2011). Recently, a population of waterhemp (*Amaranthus*

*tuberculatus*) resistant to 2,4-D and less sensitive to dicamba compared to several sensitive populations was reported (Bernards et al. 2012). Uptake and translocation of 2,4-D was equivalent in the resistant and sensitive populations, but metabolism was faster in the resistant population (Leibhart et al. 2014).

Further research into the mechanisms of resistance to 2,4-D in resistant dicot weed populations, and studies on the genetics and inheritance of resistance will provide a better understanding of these weeds and how to prevent the development of more resistant populations. Investigating what is the major mechanism of resistance to 2,4-D (uptake, translocation, metabolism, target site mutation, etc.) will allow researchers to focus on one specific mechanism. For example, if metabolism was hypothesized to be the main mechanism of resistance, the metabolic fate of 2,4-D in the resistant population could be compared with a naturally tolerant species like corn. This research could determine if 2,4-D is being metabolized similarly or if the resistant population has a greater abundance or activity of a certain detoxifying enzyme compared to the sensitive population. Determining the main resistance mechanism will also allow researchers to investigate how to best manage the resistant population and how the resistance trait may spread. Weed populations that are dioecious and therefore obligate out-crossers, such as waterhemp and Palmer amaranth (*Amaranthus palmeri*), are able to spread resistance traits via pollen and seed flow to rapidly promote an infestation (Steckel 2007). Investigating the mechanism of 2,4-D resistance and how the trait is linked genetically (dominant or recessive gene, single or multiple genes, associated with phenotypic trait or fitness penalty, etc.) with gene flow may allow researchers to understand how far and/or quickly the resistance trait can spread or how the resistance mechanism developed initially.

**Attributions**

The material presented in Chapter 2 is currently in the process of being submitted for publication in *The Journal of Agricultural and Food Chemistry* and Chapter 3 is in the process of being submitted for publication in *Weed Science* with contributions by Josh Skelton, David Simpson, Mark Peterson, and Dean Riechers. The experimental design was developed by all authors and research was conducted utilizing assistance and radiolabeled methods developed by Rong Ma. Assistance with HPLC-method development was provided by Dr. Anatoli Lygin. LC-MS/MS was conducted by Furong Sun at the University of Illinois Mass Spectrometry Lab, and LC-MS/MS data analysis was conducted by Josh Skelton with assistance from Furong Sun. Sample preparation, oxidation, and other tasks were assisted by Jacquie Janney, Brittany Janney, and Erin Lemley. Data analysis was conducted by Josh Skelton with recommendations from Adam Davis.

The material presented in Chapter 4 is currently in the process of being submitted for publication in *Crop Protection* with contributions by Josh Skelton, Rong Ma, and Dean Riechers. Experimental design was developed by Josh Skelton and research was conducted using greenhouse methods and techniques developed by Rong Ma. Planting, watering, and weighing plant samples were assisted by Brittany Janney and Erin Lemley.

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## CHAPTER 2

## BIOKINETIC AND METABOLIC FATE OF 2,4-D IN 2,4-D-RESISTANT SOYBEAN (GLYCINE MAX)

### Introduction

The Enlist™ Weed Control System provides resistance to the herbicide 2,4-dichloro-phenoxyacetic acid (2,4-D) in several crops including soybean (*Glycine max*), corn (*Zea mays*), and cotton (*Gossypium hirsutum*). Resistance to 2,4-D is derived from insertion of a transgene belonging to the bacterial enzyme class of aryloxyalkanoate dioxygenases (AAD), which cleave 2,4-D to the nonherbicidal metabolite, dichlorophenol (DCP) (Wright et al. 2010). During the development of Enlist soybean, crop injury was occasionally observed (Figure 2.1), but this injury did not occur when 2,4-D choline was applied at a field-use rate. However, when equivalent rates of 2,4-D were applied as Enlist Duo (premix of 2,4-D choline, glyphosate, and adjuvants) or 2,4-D choline tank-mixed with glyphosate, necrotic spots rapidly formed on treated leaves, which is not characteristic of synthetic auxin herbicides. Injury was not observed in new growth and did not change grain yield (data not shown) or yield components (Robinson et al. 2015) but was unexpected because previous *in vitro* kinetics assays displayed very rapid 2,4-D metabolism by AAD-12 (Wright et al. 2010).

The current research was developed to gain an understanding of the biokinetic and metabolic fate of 2,4-D in sensitive and resistant soybeans, and mechanisms for why the injury in Enlist soybean occasionally forms. The metabolic fate of 2,4-D in soybean and other sensitive plants has been determined (Chakanikov et al. 1976; Davidonis et al. 1978; Feung et al. 1971, 1973, 1975; Hamilton et al. 1971; Loos 1969; Montgomery et al. 1971; Robertson and Kirkwood 1970; Shroder and Collins 2002) , and uptake and translocation has previously been measured in several plants including soybean (Barrier and Loomis 1957; Davidonis et al. 1978; Hall et al. 1982; Hauser 1955) , but none of these studies

compared 2,4-D-resistant and sensitive soybean varieties. Previous research measured the effect of foliar-applied 2,4-D at reduced rates to simulate off-target injury (Johnson et al. 2012; Kelley et al. 2005; Sciumbato et al. 2004; Slife 1956; Wax et al. 1969) but measuring uptake, translocation, and metabolism of 2,4-D in Enlist and non-transformed (NT) soybean varieties will provide unique information that has not been previously reported.

The objectives of this research were to determine the uptake, translocation, and metabolism of 2,4-D in Enlist and NT soybean varieties. The effect of glyphosate and the adjuvant from Enlist Duo on these three biokinetic factors, along with the differences between Enlist and NT varieties, will be measured. Potential options were examined for reducing or eliminating crop injury. Research hypotheses were: (1) uptake of 2,4-D between the two varieties should be equivalent, but different treatments may affect uptake levels; (2) the Enlist variety will have greater rates of 2,4-D metabolism than the NT variety, which may directly alter translocation; (3) the 2,4-D metabolic pathway in Enlist soybean will differ from NT soybean but should match DCP metabolism; (4) injury to Enlist soybean is derived from increased foliar uptake rather than alteration of metabolism by glyphosate.

**Materials and Methods**

*Uptake and Translocation Assay*

Enlist and NT soybean lines (provided by Dow AgroSciences, Indianapolis, IN 46268) were grown to the V2 growth stage in a growth chamber (16 hour day/8 hour night, 28˚C/26˚C, and photosynthetically active radiation was 490 µmol photons/m$^2$s). The Enlist and NT soybeans are isogenic lines (varieties) that share the same genetic background with the only difference being the 2,4-D-resistance trait. The V2 growth stage was selected because it is the growth stage at which most injury was displayed in field studies (data not shown). Three different experiments were conducted with the

focus of the first being on the effect of the adjuvant from Enlist Duo, and the second focused on the impact of glyphosate and using the premix Enlist Duo product or a hand-mixture of the ingredients on uptake (Table 2.1). A third experiment was conducted to measure the uptake of 2,4-D salt and 2,4-D ester using the adjuvant from Enlist Duo (Table 2.1).

At V2, the plants were treated with herbicide treatments (Table 2.1) 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 applying the radiolabeled herbicide treatments. Twenty µM [Uniformly Ring-Labeled (URL)-$^{14}C$]-2,4-D solutions (specific activity 1.4 GBq/mmol) were the same as the whole-plant spray solutions but spiked with radiolabeled 2,4-D (Table 2.1). Herbicide solutions were applied using a glass syringe (Hamilton Co., Reno, NV 89502) to deliver 10 µL (100 Bq) divided into 33 droplets to the treated trifoliate leaf. Droplets were equally divided among the three leaflets. The second true leaf was used as the treated leaf and was noted with a marker for clear recognition during harvesting. Some treatments resulted in the formation of necrotic spots where the radiolabeled solution had been applied to soybean leaves (similar to the injury displayed in field studies, Figure 2.1) but injury did not result from unlabeled herbicide pre-treatments listed in Table 2.1 (data not shown).

After the radiolabeled solution was applied, the plants were returned to the growth chamber until sample collection at 1, 3, 6, and 24 hours after application (HAA). At sampling, the plant was divided into three parts: the treated leaf, above the treated leaf portion, and below the treated leaf portion. The treated leaf was the entire trifoliate leaf to which the radiolabeled herbicide solution was applied. The above and below the treated leaf portions were determined by dividing the plant into two halves based upon where the petiole of the treated leaf connects to the stem. The treated leaf was immediately rinsed in 3 mL of a water:methanol solution (80:20, v/v) for 45 s to remove any remaining

$^{14}C$-2,4-D material from the leaf surface in a scintillation vial. The methanol rinse solution was then analyzed via liquid scintillation spectrometry (LSC, Model 1900 TR, Packard Instrument, Meriden, CT 06450) to determine the amount of $^{14}C$-2,4-D left on the surface. The treated leaf, above, and below the treated leaf portions were allowed to air dry before the portions were combusted for 4 min in a biological oxidizer (Model OX-500, R.J. Harvey Instrument Corp., Hillsdale, NJ 07462) and analyzed via LSC to quantify the amount of $^{14}C$-labeled material within each plant portion. Total radioactive material recovery was determined by calculating the sum of the amount of $^{14}C$-material recovered from each step and averaged 90% for all experiments.

Enlist and NT soybeans were analyzed with photostimulated luminescence (PSL) to qualitatively determine where the $^{14}C$-material is located in the plant at 24 HAA. Enlist and NT soybean plants were treated in a similar fashion as in the whole-plant assays, but a higher amount of $^{14}C$-2,4-D was used (416 Bq). After air-drying, samples were placed on a phosphorimage exposure cassette (Molecular Dynamics) for 48 h before analyzing by PSL (Typhoon 9400 Variable Mode Imager, Amersham Biosciences, Buckinghamshire, England). Dried samples were oxidized to determine amount of radiolabeled material in each portion as a reference to the phosphorimage.

*Metabolism Assay*

Due to differences in 2,4-D uptake among treatments measured in preliminary whole-plant assays and observed injury with certain treatments, an excised leaf assay was developed to normalize the amount of $^{14}C$-2,4-D within the plant and produce more accurate metabolism-degradation results. The method used for the excised-leaf assay utilized similar methods as in previous research with a dicot weed, *Amaranthus tuberculatus* (Ma et al. In press). Both soybean varieties were grown in a growth chamber to the V2 growth stage to be consistent with the whole-plant assay, and the second leaf was

used to measure 2,4-D metabolism. The treated leaf was cut from the stem at the base of the petiole, and the cut petiole was placed under water and cut again to ensure that air was not trapped in the vascular tissue. The excised leaf assay was divided into three stages: (1) 0.1 M Tris buffer pH 6.5, (2) herbicide solution, and (3) water. After removing the leaf from the plant, the leaf was inserted into a 1.5 mL Eppendorf tube containing 200 µL of the buffer solution for 0.5 h to acclimate to the buffer. The leaf was then transferred to a new Eppendorf tube containing 100 µL (4.2 kBq) of the herbicide solution for 0.5 h. The herbicide solution was 25 µM $^{14}$C-2,4-D (specific activity 1.4 GBq/mmol) in 0.1 M Tris buffer pH 6.5, and the treatments were 2,4-D only and 2,4-D + glyphosate (36 mM). After 0.5 h, almost all of the herbicide solution had been absorbed and the remaining amount was quantified using LSC. Finally, the leaves were transferred to an Eppendorf tube containing 1.5 mL of distilled water and would remain in the tubes until sample collection at 1, 3, 6, 12, and 24 HAA. Distilled water was added throughout this stage to ensure that the leaf had a constant supply of water and was not stressed. Water was used instead of half-strength MS salt solution (Ma et al. In press) because soybean leaves developed injury after 24 hours when the MS solution was used.

At each sampling time, the leaf was removed from the solution and processed to extract $^{14}$C-material. The leaf was frozen with liquid nitrogen, homogenized with a glass rod, and treated with 14 mL of a water:acetone solution (10:90, v/v) for 16 h at -4˚C. Following acetone extraction, samples were centrifuged at 12,000*g* for 10 min resulting in two distinct phases, a solid (non-extractable) and liquid (extractable) portion. The non-extractable portion was allowed to air dry before oxidization to determine the amount of $^{14}$C-material as bound residue. The liquid portion was analyzed using reverse-phase high pressure liquid chromatography (RP-HPLC) to determine the metabolism of $^{14}$C-2,4-D. Samples for RP-HPLC analysis were created by concentrating samples at 40˚C until a final volume of 0.5 mL was reached with a rotary evaporator (Rotavapor R-200, BÜCHI, Flawil, Switzerland 9230).

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 LSC. HPLC samples were normalized so 38.5 Bq was injected. Recovery of $^{14}C$-material ranged from 92 to 98% for all experiments.

Reverse-phase HPLC was performed on a Perkin Elmer Flexar LC HPLC (Model N2910401, Perkin Elmer, Akron, OH 44311) with an Altima $C_{18}$ column (4.6 X 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 (25 min total). Radiolabeled compounds were detected with a β-RAM Radio-HPLC Detector (Model 4, Lab Logic, Brandon, FL 33511) and Ultima-Flo M cocktail (Perkin Elmer). [URL-$^{14}C$]-2,4-D displayed a retention time of 21 min. Figure 2.2 shows a typical chromatogram of Enlist and NT soybean 2,4-D metabolism at 1 and 24 HAA using these methods.

*Assays of 2,4-D Acid, 2,4-D Ester, and DCP Metabolism*

The excised-leaf assay was used to investigate the metabolism of 2,4-D acid compared with 2,4-D ester to determine the metabolic fate of 2,4-D ester, a proherbicide, in Enlist soybean. Using the methods previously described, an excised-leaf assay using 25 µM $^{14}C$-2,4-D acid (specific activity 1.4 GBq/mmol) and $^{14}C$-2,4-D-ethylhexyl ester (EHE) (specific activity 1.5 GBq/mmol) at the sampling points of 1, 3, and 6 HAA was developed to investigate any qualitative differences in metabolism in Enlist soybean. Another assay utilized 25 µM $^{14}C$-DCP (specific activity 1.2 GBq/mmol) to measure DCP metabolism in Enlist and NT soybeans at 1, 3, 6, 12, and 24 HAA. DCP was utilized because it is the first metabolite formed during 2,4-D metabolism in Enlist soybean by the AAD-12 enzyme (Wright et al.

2010) and would show if DCP is metabolized similarly between soybean varieties regardless of the transgene. Samples generated using the excised-leaf assays were analyzed by HPLC as previously described to determine the metabolite(s) formed in Enlist soybean, but with modifications as outlined below.

Reverse-phase HPLC was performed using the same system as the previous excised-leaf assays but utilized a different column and mobile phase. A ThermoScientific $C_4$ Column (Hypersil Gold C4, 4.6 x 250 mm, 5 µm) was used to analyze samples. Eluent A was 0.1% (v/v) formic acid in water and eluent B was acetonitrile. The 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, [URL-$^{14}$C]-2,4-D EHE displayed a retention time of 30 min, and [URL-$^{14}$C]-DCP displayed a retention time of 14 min.

*LC-MS/MS Analysis of 2,4-D Metabolites in Enlist Soybean*

Enlist soybean leaves were treated with $^{14}$C-2,4-D and collected at 24 HAA utilizing the same methods as the excised-leaf assay. To increase the concentration of 2,4-D metabolite(s) prior to analysis, four leaf samples from different soybean plants were combined before extraction and concentration. The LC-MS/MS system consisted of an HPLC separation module (Waters Alliance 2795) equipped with an electrospray ionization mass spectrometer (Waters QuattroUltima) in the positive ion mode $(M+H)^+$. Samples were analyzed using a 65 min linear gradient from 100% A to 100% B. Eluent A was 95% water:5% acetonitrile and eluent B was 5% water:95% acetonitrile. Samples were analyzed using a ThermoScientific $C_4$ Column (Hypersil Gold $C_4$, 4.6 x 250 mm, 5 µm). The retention times of the two metabolites analyzed using this gradient were 12 min and 16 min 48 s. Data were processed using Waters Mass Lynx software (version 4.1) to determine parent and fragment ions.

*Statistical Methods*

Treatments were arranged in a completely randomized design and data from each independent experiment were combined and analyzed. All experiments were replicated twice and contained three biological replications for each treatment combination. Uptake and metabolism data were analyzed using nonlinear regression methods using the Michaelis-Menten model (inverse hyperbolic curve; Equation 1) (Kniss et al. 2011) 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, $Y$ represents the percentage of 2,4-D uptake or metabolism at time $t$, $\alpha$ is the parameter that estimates the maximum amount of $Y$, and $\beta$ is the parameter that estimates the $t$ to reach 50% $\alpha$. Parameters of different models were compared using pairwise differences ($\alpha$=0.05) with the *compParm* function in R. The translocation and non-extractable data were analyzed with SAS (SAS version 9.4, SAS Institute Inc., Cary, NC 27513) using Proc Mixed in a split-plot design with the whole-plot being each time point and the sub-plot 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, so results were pooled over both experimental replications for all data.

**Results and Discussion**

*Uptake and Translocation of 2,4-D in Enlist and NT Soybeans*

A difference in the amount ($U_{max}$; P=0.3729, 0.6159) or rate ($U_{50}$; P=0.6102, 0.5040) of 2,4-D uptake between the Enlist and NT soybean varieties was not measured in either experiment. There was a significant difference in the parameters by the various treatments (P=0.0459, 0.0094; P=<0.0001,

<0.0001). In the first experiment, the treatments with the adjuvant (Adj.) from Enlist Duo (2,4-D + Adj. and Enlist Duo) had greater 2,4-D uptake than the treatments without the adjuvant (2,4-D only and 2,4-D + glyphosate) (Table 2.2). The $U_{max}$ for the 2,4-D + Adj. treatment was greater than the Enlist Duo treatment (Table 2.2). The Enlist Duo treatment had the fastest rate of uptake (lowest $U_{50}$), taking 0.2 h for 50% 2,4-D uptake to occur compared to 0.6 and 0.7 h for the other three treatments. At 1 HAA, the Enlist Duo treatment had 78% 2,4-D uptake, which was greater than the 2,4-D + Adj. treatment (56%), but uptake was equivalent between the two treatments from 3 to 24 HAA. The two treatments with the Enlist Duo adjuvant had greater uptake than the two treatments without the adjuvant at all time points. In the second experiment, Enlist Duo had a lower $U_{max}$ than the other two treatments, 2,4-D + Adj. and 2,4-D + glyphosate + Adj., but faster rate of uptake (Table 2.2). The $U_{50}$ value for Enlist Duo was 0.3 h compared to 1.2 h for 2,4-D + Adj. and 1.1 h for 2,4-D + glyphosate + Adj. Enlist Duo had 71% 2,4-D uptake at 1 HAA which was greater than the other two treatments (42% and 47%), but uptake levels were equal between all three treatments from 3 to 24 HAA (Fig. 2.3).

There was a significant difference between soybean varieties in translocation of $^{14}C$-material. The Enlist variety had more $^{14}C$-material remaining in the treated leaf compared to the NT variety in both studies (P=0.0472, <0.0001). Less radiolabeled material remained in the treated leaf of the NT variety at 6 and 24 HAA (Table 2.3). In both varieties, a difference in the movement of $^{14}C$-material to the above or below the treated leaf portion of the plant was not measured (Table 2.3). Figure 2.4 shows a phosphorimage of Enlist and NT soybeans at 24 HAA. The location of $^{14}C$-material is heavily concentrated in the treated leaves (Fig. 2.4 A and B; 85% and 95%), but more $^{14}C$-material is located in other plant portions in the NT soybean (Fig. 2.4 C; 15%) compared to Enlist soybean (Fig. 2.4 D; 5%) matching the results from the whole-plant assays.

*Movement of 2,4-D Within the Treated Leaf in Enlist Soybean*

The formation of necrotic spots in Enlist soybean may be related to an increase in ethylene due to the high initial concentrations of 2,4-D. Ethylene is a gaseous hormone involved in many developmental processes such as fruit ripening, leaf senescence, and mediates plant respondes to pathogen attack (Boller 1991; Ciardi et al. 2001; Knoester et al. 1998; Pennickx et al. 1998). For example, *de novo e*thylene production induces plant defense mechanisms including abscission or a hypersensitive response after infection by a pathogen or wounding by an insect (Boller 1991; Ciardi et al. 2001; Knoester et al. 1998; Pennickx et al. 1998). Hypersensitive response is defined as cell death in order to prevent further infection or injury from the pathogen or pest resulting in necrotic spots around the infection site (Boller 1991; Ciardi et al. 2001; Knoester et al. 1998; Pennickx et al. 1998). Since auxin herbicides induce ethylene in sensitive plants through an auxin-stimulated pathway (Grossman 2010) it follows that 2,4-D not initially metabolized in Enlist soybean may induce ethylene production and trigger a rapid hypersensitive response.

A phosphorimage of Enlist soybean with and without injury derived from rapid 2,4-D uptake was generated to determine if radiolabeled material was trapped in the necrotic spots (Fig. 2.5). The concentration of radiolabeled material was highest where the solution was applied in both treatments, but more movement away from the site of droplet application was observed with the Enlist Duo treatment (Fig. 2.5). This image shows that injury to Enlist soybean caused by the Enlist Duo formulation did not impede movement of radiolabeled material within the leaf. Measuring ethylene levels around these necrotic lesions and determining whether 2,4-D is metabolized or trapped as the free acid in the lesion may explain if the injury observed in Enlist soybean is related to a hypersensitive response and/or auxin-stimulated ethylene production.

*Comparison of 2,4-D Acid and Ester Uptake in Enlist Soybean*

The $U_{max}$ for both formulations were equal (P=0.6785), but the rate of 2,4-D uptake ($U_{50}$) with the ester formulation was much more rapid (P=0.0055) than 2,4-D acid. The $U_{50}$ value for the ester formulation was 0.08 h compared to 1.6 h for the acid formulation with both treatments containing glyphosate and the adjuvant from Enlist Duo. At 1 HAA, herbicide uptake was 85% with the ester formulation compared to 39% with the acid. Uptake with the ester formulation was greater than the acid at 1 and 3 HAA but equivalent at 6 and 24 HAA. Greater 2,4-D ester uptake was expected due to its lipophilic nature, resulting in greater movement through the cuticle into the plant. Even with the increase in 2,4-D uptake, Enlist soybean plants treated with 2,4-D ester did not develop injury in treated leaves as observed with 2,4-D choline (Fig. 2.6).

The adjuvant from Enlist Duo significantly increased the uptake of 2,4-D in soybean, and the rate of uptake with Enlist Duo was much faster than other treatments. Enlist Duo, a premixed product, had faster uptake compared with hand-mixing the individual components of Enlist Duo and treatments containing the same adjuvant. Glyphosate did not alter 2,4-D uptake in soybean. Additional 2,4-D salt formulations should be compared to determine if the choline salt is inducing injury or if injury is independent of the salt formulation. Less $^{14}C$-material translocated out of the treated leaf in Enlist soybean compared to NT soybean. This may be linked to rapid detoxification of 2,4-D and storage of metabolites in the treated leaf of Enlist soybean compared to NT soybean, which are not able to detoxify 2,4-D and therefore allows herbicide translocation in the phloem. In this case, NT soybean are responding similar to a sensitive weed compared to the Enlist variety, which resembles a naturally-tolerant grass species (Ashton 1958; Fang and Butts 1954).

The use of 2,4-D ester instead of 2,4-D choline did not result in injury to Enlist soybean. Levels of 2,4-D ester uptake were greater than 2,4-D choline, especially immediately after herbicide application, but injury did not develop. The difference in metabolism of 2,4-D ester compared to 2,4-D acid may explain the lack of injury in Enlist soybean.

*Metabolism of 2,4-D in Enlist and NT Soybeans*

There was a significant difference in the rate ($M_{50}$; P=<0.0001) of 2,4-D metabolism but not the maximum amount ($M_{max}$; P=0.7889) modeled between the Enlist and NT varieties (Table 2.4). The Enlist variety required 0.2 h to metabolize 50% of the 2,4-D compared to 26.8 h for the NT variety and had greater 2,4-D metabolism at all sampling points. At 1 HAA, Enlist soybean had metabolized 76% of the 2,4-D compared to 4% in NT soybean (Fig. 2.7). The amount of metabolism by the NT variety increased with time, but at 24 HAA, the amount of 2,4-D metabolized by the NT variety was 58%, which was significantly less than the Enlist variety (95%; Fig. 2.7). A significant difference was not detected in either metabolism parameter when glyphosate was included. The amount (P=0.4920) and rate (P=0.2381) of 2,4-D metabolism between the 2,4-D only and 2,4-D + glyphosate treatments were similar.

The formation of non-extractable $^{14}C$-material was significantly greater in Enlist soybean (59%) compared to NT (30%; P=<0.0001). A significant variety by hour interaction occurred (P=0.0448). At all time points, the amount of non-extractable $^{14}C$-material was greater in the Enlist variety compared to NT. At 1 HAA, the amount was 25% in NT compared to 56% in Enlist soybean, but the magnitude of difference between the two varieties decreased slowly, and at 24 HAA the amount in NT was 38% and 60% in Enlist soybean.

*The Metabolism of 2,4-D Acid and Ester in Enlist Soybean*

By 6 HAA, the metabolism of 2,4-D acid and 2,4-D ester resulted in the same qualitative results, but differences were apparent at 1 and 3 HAA (Fig. 2.8). At 1 HAA, the amount of 2,4-D acid present in Enlist soybean treated with 2,4-D acid was minimal. In contrast, the amount of 2,4-D ester present in plants treated with 2,4-D ester was much larger (20%; Fig. 2.8 A), and free 2,4-D acid was not detected in plants treated with 2,4-D ester at any time. Both acid and ester resulted in a common metabolite (retention time 15 min), but 2,4-D acid-treated Enlist soybean formed a second metabolite (retention time 17 min; Fig. 2.8 B and D). The amount of the common metabolite (15 min) at 1 HAA was greater in 2,4-D ester treated plants (15%) compared to 2,4-D acid (5%). At 3 HAA, the amount of the common metabolite was equal in both treatments, and the concentration of the second metabolite (17 min) formed in 2,4-D acid treated plants was reduced. After 6 h, plants treated with either 2,4-D acid or ester formed only the common metabolite (15 min), and the amount of this compound was equal in both treatments (Fig. 2.8 E and F). There was a significant difference between 2,4-D ester and acid in non-extractable $^{14}$C-material in Enlist soybean ($P=<0.0001$). The overall amount was greater with 2,4-D ester (71%) compared to 2,4-D acid (43%). A significant treatment by hour interaction was not detected ($P=0.1633$), but the amount of non-extractable $^{14}$C-material was larger at all time points with 2,4-D ester.

*DCP Metabolism in Enlist and NT Soybeans*

DCP metabolism by Enlist and NT soybeans was similar. DCP was not detected as a metabolite at any sampling time point in either variety, and by 24 HAA, both varieties had formed three distinct metabolites (Fig. 2.9 A and B). The rapid metabolism of DCP matches results from previous studies (Laurent et al. 2006, 2007; Pascal-Lorber et al. 2003). The three metabolites formed at equal amounts

between varieties (Fig. 2.9 A and B). This indicates that DCP is metabolized similarly between Enlist and NT soybeans, and that naturally occurring enzyme(s) in soybean can metabolize DCP. The same three metabolites formed in Enlist soybean treated with 2,4-D at 24 HAA (Fig. 2.9 C) but not in NT soybean (Fig. 2.9 D). Since 2,4-D is rapidly converted to DCP by the AAD-12 enzyme in Enlist soybean (Wright et al. 2010), the initial metabolites formed with 2,4-D as substrate are the same when using DCP as substrate. In NT soybean, however, 2,4-D was still present at 24 HAA along with three other metabolites (Fig. 2.9 D). The first metabolite (peak retention time of 3 min 54 s) was also detected in the metabolite profile of DCP in NT soybean, but peaks at 13 min 7 s and 10 min 15s were not present in any other treatment (Fig. 2.9 D). These peaks may contain an amino acid or glucose conjugate of 2,4-D, which were measured in previous metabolism studies (Chakanikov et al. 1976; Davidonis et al. 1978; Feung et al. 1971, 1973, 1975; Hamilton et al. 1971; Loos 1969; Montgomery et al. 1971; Robertson and Kirkwood 1970; Shroder and Collins 2002). Enlist and NT soybean did not differ in the amount of DCP non-extractable $^{14}$C-material (P=0.5215) or variety by hour interaction (P=0.3183). The overall amount in NT soybean was 79% and 78% in Enlist soybean.

*Metabolites Formed in 2,4-D Metabolism in Enlist Soybean*

Even though three metabolites were detected in the metabolism of 2,4-D and DCP by Enlist soybean (Fig. 2.9), only two were analyzed by LC-MS/MS because the concentration of the third was too low. The first metabolite (M1) with a retention time of 16 min 48 s had a parent ion at a mass-to-charge ratio (*m/z*) of 913, and the second metabolite (M2) with a retention time of 12 min displayed a parent ion at 795 *m/z* (Fig. 2.10). These metabolites, M1 and M2, correspond with the HPLC peaks at 8 min 52 s and 5 min 12 s from Figure 2.9, and the peak at 3 min 53 s represents the metabolite not analyzed further due to insufficient quantity (Fig. 2.9). DCP metabolites determined in tobacco cell culture (Laurent et al. 2007) appear as intermediates to larger sugar-based conjugates in Enlist soybean (Fig.

2.10). Metabolites observed in tobacco and Enlist soybean are 2,4-dichlorophenol-(6-O-pentosyl)-glucoside (DCP-PG; 458 *m/z*) and 2,4-DCP-glycosyl-pentosyl-glucoside (DCP-GPG; 616 *m/z*). DCP-PG was discovered in the mass spectrum of M1 and DCP-GPG was identified in M2 (Fig. 2.10). In Enlist soybean, the DCP-sugar conjugates are processed further by adding more glucose and/or pentose units compared to previously determined metabolites (Laurent et al. 2006, 2007; Pascal-Lorber et al. 2003). Figure 2.11 depicts the proposed metabolism of 2,4-D in Enlist soybean, which includes the metabolites identified in previous research (Laurent et al. 2007) and recovered metabolites in this research, M1 and M2.

Metabolism of 2,4-D was more rapid in Enlist than NT soybean, and glyphosate did not affect 2,4-D metabolism. The development of non-extractable $^{14}C$-material was greater in Enlist soybean. The increased level of non-extractable material correlates with the limited translocation of 2,4-D measured in Enlist soybean in the whole-plant assays (Table 2.3), and rapid metabolism of 2,4-D in the excised-leaf assay (Table 2.4). Enlist soybean plants appear to completely detoxify 2,4-D and permanently store the metabolite(s) in the treated leaf, which precludes translocation of 2,4-D acid to new leaves and meristems. By comparison, NT soybean do not rapidly detoxify 2,4-D, therefore allowing 2,4-D acid to become trapped in the phloem and translocate throughout the entire plant. Enlist soybean plants are thus reacting similarly as naturally-tolerant plants in the overall detoxification and storage of 2,4-D (Loos 1969). The excised-leaf assays were able to replicate previous *in vitro* kinetic studies indicating that Enlist soybean plants are resistant to 2,4-D at a larger scale than previously utilized (Wright et al. 2010)

The metabolism of 2,4-D acid and 2,4-D ester was different in Enlist soybean. When Enlist soybean leaves were treated with 2,4-D ester (1 HAA), 2,4-D ester was detected but free 2,4-D acid was absent. By 3 HAA, 2,4-D ester was not detected and the same metabolite was developed as plants treated with 2,4-D acid. The greater accumulation of non-extractable $^{14}C$-material in Enlist soybean leaves treated with 2,4-D ester suggests greater metabolism of 2,4-D, although further metabolic

analyses are required to identify the non-extractable $^{14}C$-material. This may be related to the reduction in concentration of 2,4-D acid immediately after herbicide application, the AAD-12 enzyme not being overwhelmed, and reduction of crop injury. The 2,4-D ester formulation is a proherbicide, or inactive form, of 2,4-D and must be converted to active 2,4-D acid by a carboxylesterase (CXE) enzyme inside the plant (Gershater and Edwards 2007). This additional step in the transformation of 2,4-D to non-toxic metabolites in Enlist soybean might reduce the risk of injury because the conversion of 2,4-D ester to 2,4-D acid may be rate limiting. As a result, this mechanism may act as a slow-release 'biochemical funnel' towards the cytosolic AAD-12 enzyme, thus reducing the rate of influx and cellular concentration of 2,4-D acid that must be detoxified. The whole-plant assay determined that uptake of 2,4-D was most rapid with the Enlist Duo treatment (Table 2.2), especially immediately after herbicide application. This rapid surge in 2,4-D concentration within the leaf cells may exceed the amount of substrate that can be effectively metabolized by AAD-12 before injury develops. Even though the foliar uptake of 2,4-D ester is greater than 2,4-D acid, the AAD-12 enzyme and soybean are not altered because the proherbicide must be converted to 2,4-D acid before any herbicidal response can occur. We hypothesize that the metabolic conversion of 2,4-D ester to 2,4-D acid (catalyzed by CXE enzymes) is rate limiting, which allows the AAD-12 enzyme to efficiently metabolize 2,4-D and decrease the risk of injury to soybean. Comparing CXE enzyme activity and kinetics with 2,4-D ester (Gershater et al. 2006, 2007) with that of the AAD-12 enzyme and 2,4-D acid may help to clarify these potentially rate-limiting steps in the overall metabolic pathway of 2,4-D ester in Enlist soybean.

DCP is metabolized in the same manner in both Enlist and NT soybeans. The rapid conversion of DCP to other metabolites indicates that naturally occurring enzymes are able to metabolize DCP after 2,4-D is metabolized by the AAD-12 enzyme in Enlist soybean, and is in accord with previous research on DCP metabolism in transgenic and non-transgenic cotton (Laurent et al. 2006). When Enlist soybean are

treated with 2,4-D, the three resulting metabolites are the same that form when soybean are treated with DCP. Previous studies have shown the formation of several glucose conjugates during the metabolism of DCP (Laurent et al. 2006, 2007; Pascal-Lorber et al. 2003). The metabolites identified in this study had intermediates measured in previous studies, but the formation of larger sugar-based conjugates were detected in Enlist soybean compared to previous findings (Laurent et al. 2006, 2007; Pascal-Lorber et al. 2003).

Injury to Enlist soybean occurs when 2,4-D choline, glyphosate, and the adjuvant from Enlist Duo are used either as the premix product (Enlist Duo) or as a tank-mix. This study determined that glyphosate does not influence either 2,4-D uptake or metabolism in soybean. When comparing treatments containing the same adjuvant, the Enlist Duo treatment resulted in the most rapid uptake of 2,4-D in soybean (Table 2.2). As discussed previously, the elevated cellular concentrations of 2,4-D resulting from the Enlist Duo formulation immediately after its application may exceed the metabolic capacity of the AAD-12 enzyme. Reducing the concentration of 2,4-D acid in leaf cells immediately after application by altering the Enlist Duo formulation might prevent soybean injury, but may also have several negative consequences. For example, if the adjuvant formulation in Enlist Duo was altered to reduce foliar uptake in soybean, then uptake of 2,4-D and/or glyphosate in weeds may also be lowered and reduce POST herbicide efficacy. This research demonstrated that soybean injury can be prevented if 2,4-D ester is used instead of the 2,4-D choline salt, but greater volatility and other negative aspects of 2,4-D ester (Grover et al. 1985; Marth et al. 1949; Que Hee et al. 1974) in comparison to 2,4-D choline outweigh any reductions in crop injury.

With current and future use of metabolism-based herbicide resistance traits, crop injury may occur if herbicide uptake levels exceed the metabolic capacity of the enzyme conferring resistance or if rates of herbicide metabolism are hindered, as is occasionally observed with glufosinate-resistant crops

(Krausz et al. 1999; Pline et al. 1999; Sankula et al. 1997). Future research to determine the cellular concentration of 2,4-D immediately after an application of Enlist Duo and investigate its effects on AAD-12 activity towards metabolizing 2,4-D in Enlist soybean will provide greater insight into the potential risk of crop injury in the field. Balancing the cellular concentration of 2,4-D and the maximum amount that can be metabolized by AAD-12 before injury develops should ultimately eliminate the risk of soybean injury.

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**Tables**

Table 2.1. Treatment list for whole-plant assays used to determine 2,4-D uptake and translocation in Enlist and non-transformed soybean varieties.

| Experiment 1 | Experiment 2 | Experiment 3 |
|---|---|---|
| 2,4-D choline only[a] | 2,4-D + Adj. | 2,4-D choline + Glyphosate + Adj. |
| 2,4-D choline + Adj.[b] | 2,4-D + Glyphosate + Adj. | 2,4-D Ester[e] + Glyphosate + Adj. |
| 2,4-D + Glyphosate[c] | Enlist Duo™ | |
| Enlist Duo™[d] | | |

[a] 2,4-D choline (1065 g ae/ha, 2,4-D choline 3.8L, Dow AgroSciences, Indianapolis, IN 46268).

[b] Adjuvant (Enlist Duo Adjuvant Mix, Dow Agrosciences, Indianapolis, IN 46268).

[c] Glyphosate (1120 g ae/ha, Glyphosate dimethylammonium salt 781.254 g $L^{-1}$, Dow AgroSciences, Indianapolis, IN 46268).

[d] Enlist Duo (2185 g ae/ha, Enlist Duo 3.33L, Dow AgroSciences, Indianapolis, IN 46268).

[e] 2,4-D Ester (1065 g ae/ha, Low Vol 4 Ester Weed Killer, Dow AgroSciences, Indianapolis, IN 46268).

AMS (2.5% v/v, N-PAK AMS Liquid 34%, Dow AgroSciences, Indianapolis, IN 46268) was added to all treatments.

Table 2.2. Nonlinear regression parameters for 2,4-D uptake from whole-plant assays with Enlist and non-transformed soybean varieties.

| | $U_{max}$ [a] | $U_{50}$ [b] |
|---|---|---|
| Treatment | % Uptake [c] | Hours [c] |
| 2,4-D only | 56.3 c | 0.7 a |
| 2,4-D + Adjuvant | 101.9 a | 0.7 a |
| 2,4-D + Glyphosate | 64.2 c | 0.6 a |
| Enlist Duo | 94.7 b | 0.2 b |
| 2,4-D + Adjuvant | 105.4 a | 1.2 a |
| 2,4-D + Glyphosate + Adjuvant | 104.6 a | 1.1 a |
| Enlist Duo | 93.3 b | 0.3 b |

[a] Parameter corresponding to the modelled maximum 2,4-D uptake (% recovery) of treatment.

[b] Parameter corresponding to time (hours) for treatment to reach 50% $U_{max}$.

[c] Treatments followed by the same letter within each experiment are not significantly different by Fisher's LSD Test α=0.05.

Table 2.3. Distribution of $^{14}$C-material from whole-plant assays using Enlist and non-transformed soybean varieties.

| | | Hours After Application | | | |
|---|---|---|---|---|---|
| | | 1 | 3 | 6 | 24 |
| Variety | Portion | % $^{14}$C-Material in Plant[a,b,c] | | | |
| Non-Transformed | Treated Leaf | 99.5 a | 98.8 a | 96.4 b | 93.0 b |
| | Above | 0.2 | 0.8 | 2.5 | 3.6 |
| | Below | 0.3 | 0.4 | 1 | 3.5 |
| Enlist | Treated Leaf | 99.6 a | 99.3 a | 98.9 a | 98.3 a |
| | Above | 0.1 | 0.2 | 0.4 | 0.6 |
| | Below | 0.3 | 0.4 | 0.6 | 1.2 |

[a] Significant difference between soybean varieties in amount $^{14}$C-material remaining in treated leaf (P=<0.0001).

[b] No significant difference in either variety between amount $^{14}$C-material in above or below treated leaf portions (P=0.5572).

[c] Values followed by the same letter within a time point are not statistically different at α=0.05 by Tukey's Test of Honest Difference.

Table 2.4. Nonlinear regression parameters for 2,4-D metabolism from the excised-leaf assays with Enlist and non-transformed (NT) soybean varieties.

| | | $M_{max}$ [a] | $M_{50}$ [b] |
|---|---|---|---|
| Variety | Treatment | % Metabolized[d] | Hours[c,e] |
| Enlist | | 95.2 | 0.2 b |
| NT | | 100.0 | 26.8 a |
| | 2,4-D Only | 65.6 | 0.5 |
| | 2,4-D + Glyphosate | 73.3 | 1.8 |

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

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

[c] Treatments followed by the same letter within each experiment are not significantly different by Fisher's LSD Test α=0.05.

[d] $M_{max}$ for variety and treatment not significantly different (P=0.7889; 0.4920).

[e] $M_{50}$ for variety significantly different (P=<0.0001) and treatment not significantly different (P=0.2381).

## Figures

Figure 2.1. Injury to Enlist soybean during field applications of 2,4-D choline, 2,4-D choline + glyphosate, and Enlist Duo one day after treatment. All treatments contained the adjuvant blank from Enlist Duo. Injury occurred on treated leaves of soybean treated with 2,4-D choline + glyphosate and Enlist Duo, but not with 2,4-D choline alone. Necrotic spots formed on treated leaves, but injury was not observed in new growth and had no impact on grain yield.

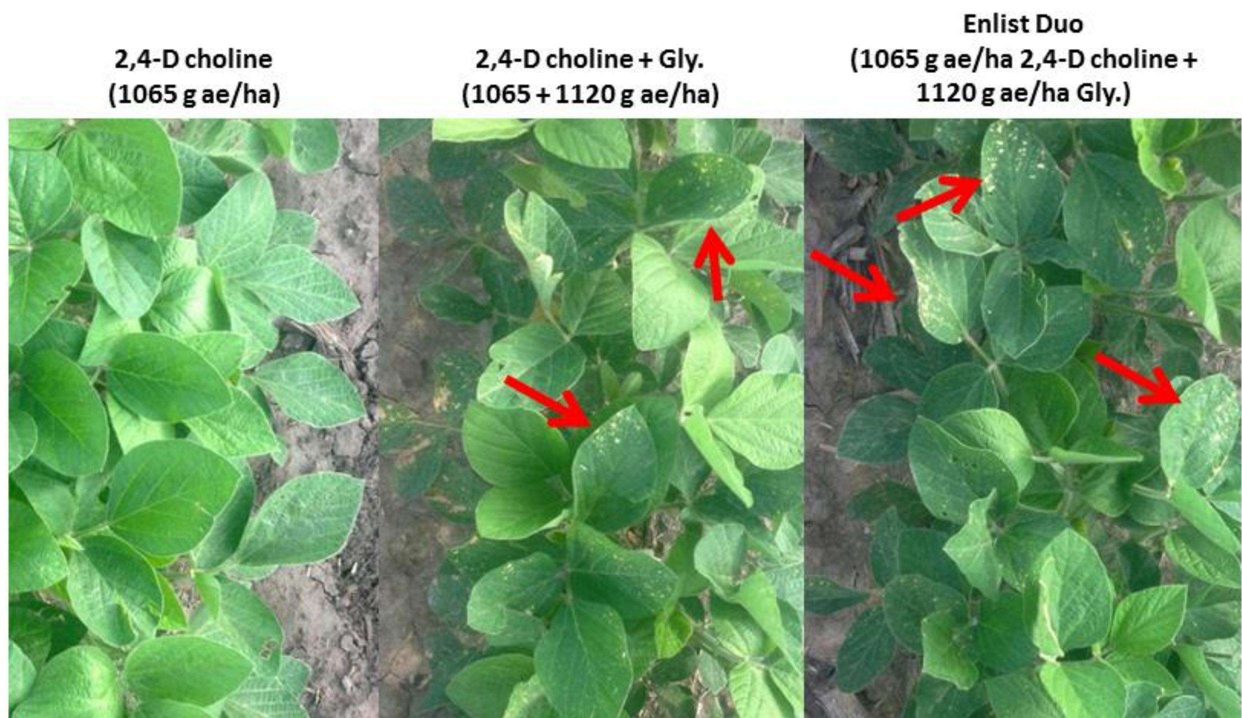


Figure 2.2. Typical HPLC radiochromatograms from the excised-leaf assay to determine the amount of parent $^{14}C$-2,4-D (retention time 21 min) present at each time point. (A) Enlist soybean at 1 HAA, (B) NT soybean at 1 HAA, (C) Enlist soybean at 24 HAA, and (D) NT soybean at 24 HAA.

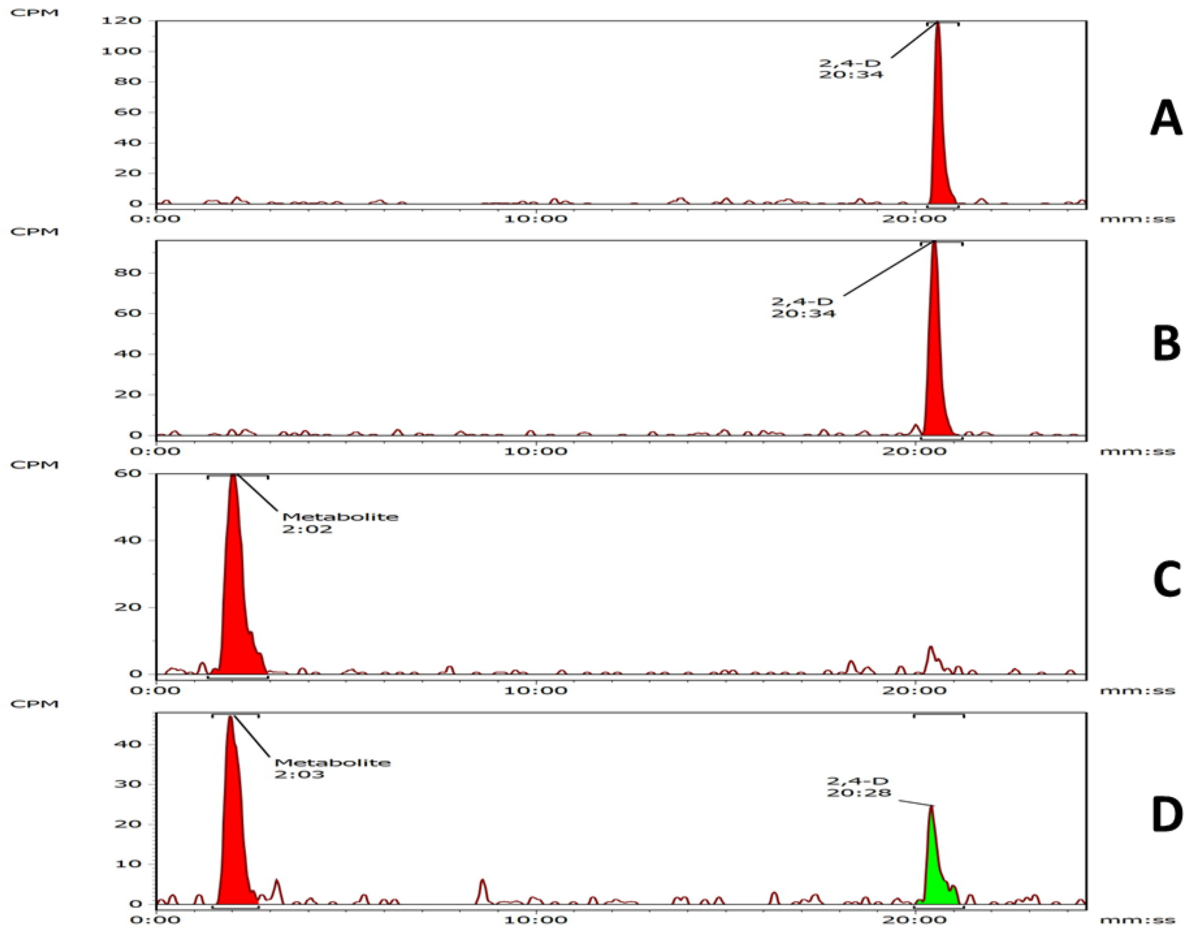


Figure 2.3. Uptake of 2,4-D among the three treatments from the second whole-plant assay with Enlist and non-transformed soybeans over the 24 h time course. Markers represent the average uptake among treatments and error bars represent the standard error of the regression model. All treatments were applied at the same rate of 2,4-D choline (1065 g ae/ha), glyphosate (1120 g ae/ha), adjuvant, and AMS (2.5% v/v) was included.

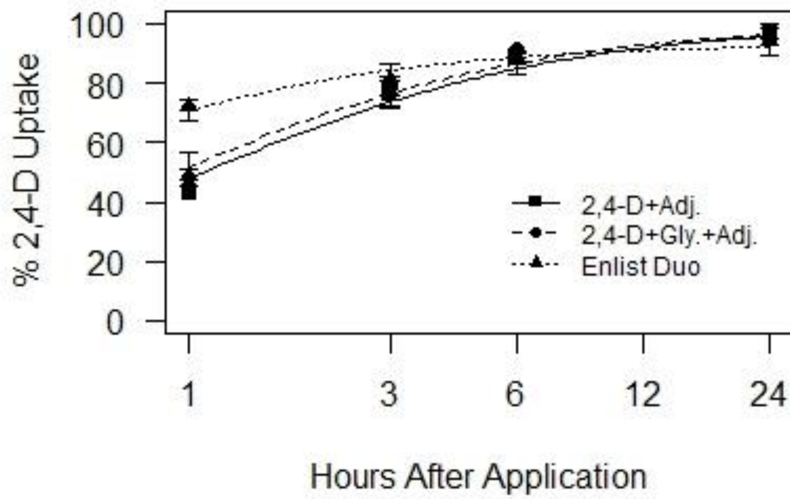


Figure 2.4. Phosphorimage and picture of Enlist and non-transformed (NT) soybeans at 24 HAA treated with $^{14}$C-2,4-D. Areas of increased intensity indicate a relatively higher amount of radiolabelled material. (A) NT treated leaf, (B) Enlist treated leaf, (C) NT above and below treated leaf portion, and (D) Enlist above and below treated leaf portion. Arrows indicate point of attachment for treated leaves. Both Enlist and NT roots were analyzed but are not visible on the phosphorimage due to amounts of $^{14}$C-material below the detection threshold. Plant portions were oxidized to measure the percent of $^{14}$C-material of total $^{14}$C-material absorbed: (A) 85%, (B) 95%, (C) 15%, and (D) 5%.

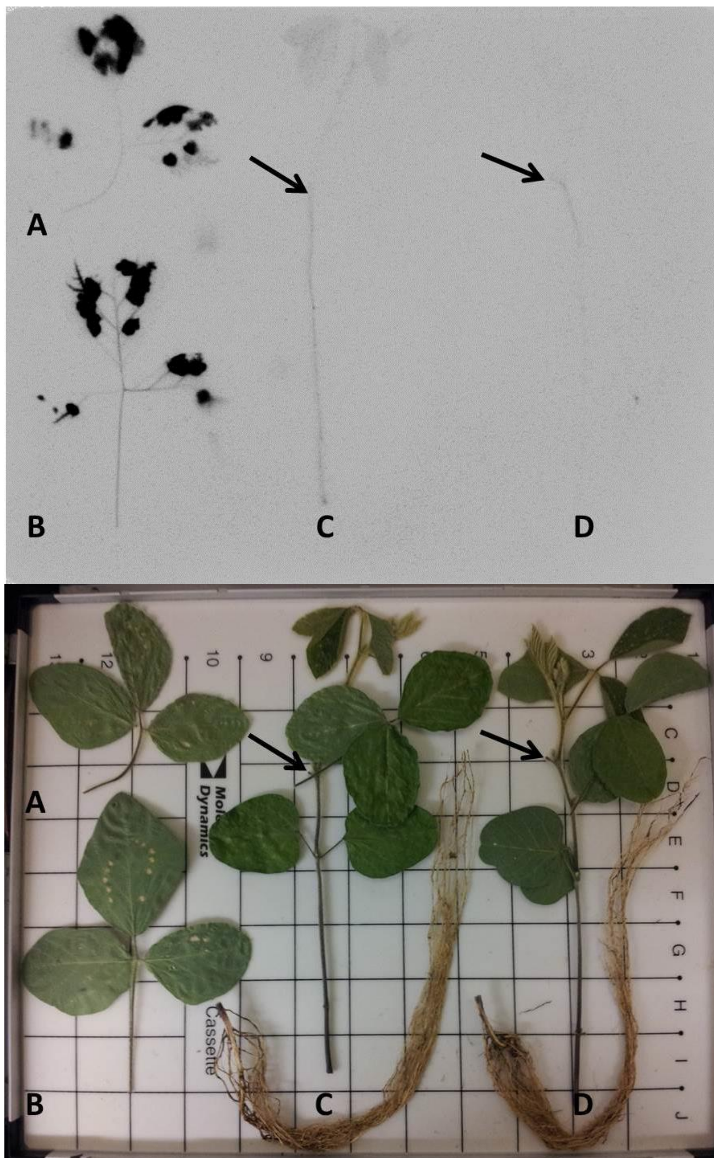
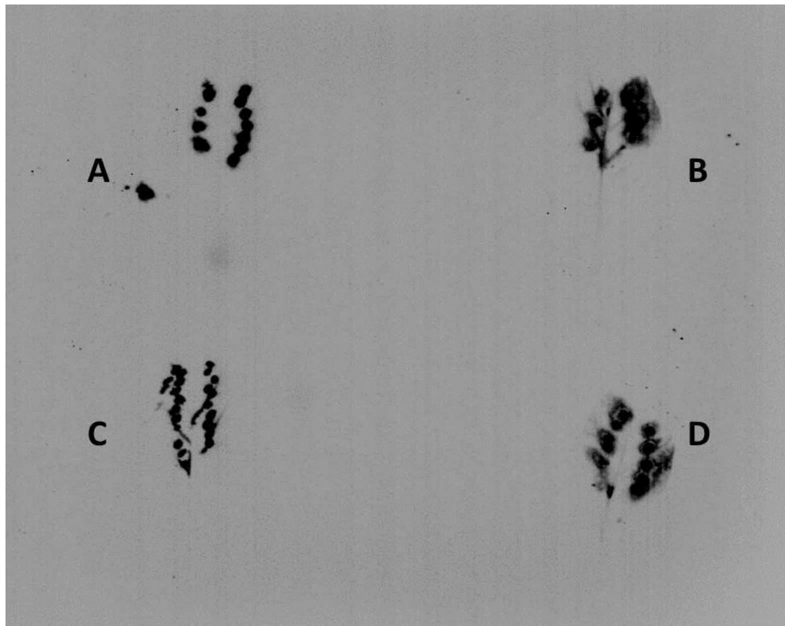


Figure 2.5. Phosphorimage and picture of Enlist soybean treated with 2,4-D + adjuvant from Enlist Duo (A and C) or Enlist Duo (B and D). Areas of increased intensity indicate a relatively higher amount of radiolabeled material. Treatments were applied as large droplets (0.6 µL, A and B) or smaller droplets (0.3 µL, C and D) to determine if the size of necrotic injury affects movement of radiolabeled material. Soybean leaf injury symptoms developed with Enlist Duo but not the 2,4-D + Adjuvant treatment.



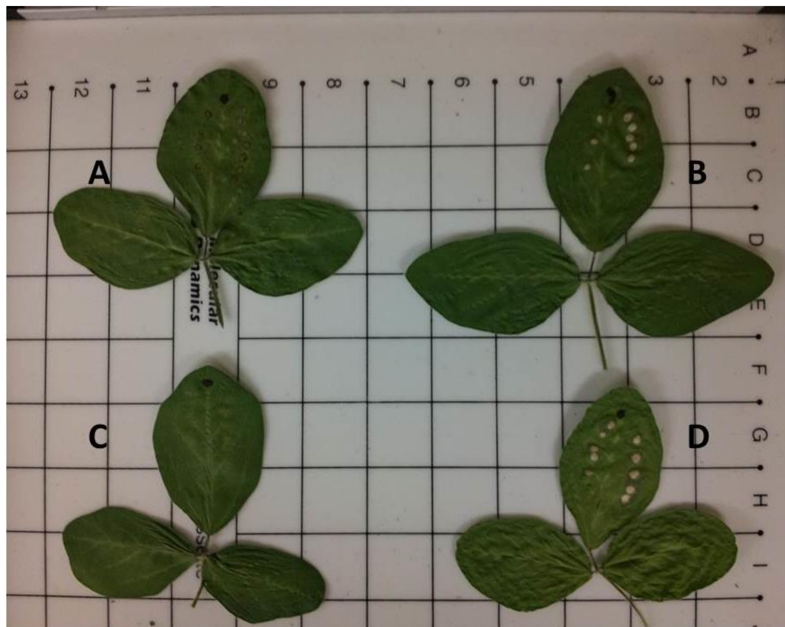



Figure 2.6. Enlist soybean at 24 HAA treated with (A) 2,4-D choline + glyphosate + adjuvant from Enlist Duo and (B) 2,4-D ester + glyphosate + adjuvant from Enlist Duo. Both formulations were applied at the same acid equivalent rate, but injury did not form on Enlist soybean treated with 2,4-D ester.

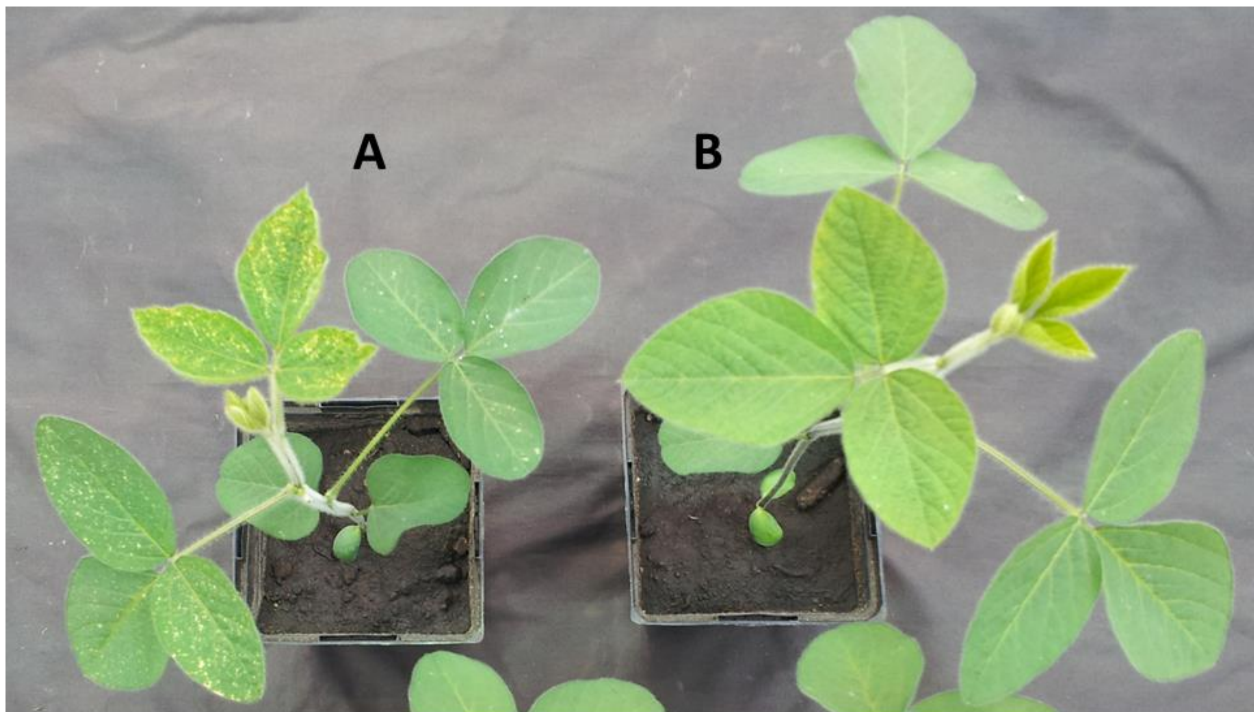


Figure 2.7. Metabolism of 2,4-D in Enlist (E) and non-transformed (NT) soybean varieties excised-leaves. Markers represent the average amount of 2,4-D metabolized and error bars represent standard error of the non-linear regression model at each time point.

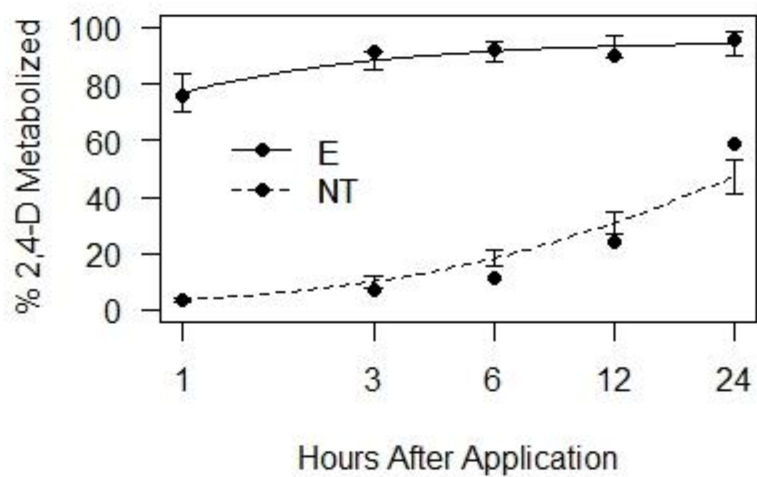


Figure 2.8. HPLC radiochromatograms measuring 2,4-D acid and 2,4-D ester metabolism in Enlist soybean using the excised-leaf assay. (A) 2,4-D ester metabolism at 1 HAA, (B) 2,4-D acid metabolism at 1 HAA, (C) 2,4-D ester metabolism at 3 HAA, (D) 2,4-D acid metabolism at 3 HAA, (E) 2,4-D ester metabolism at 6 HAA, and (F) 2,4-D acid metabolism at 6 HAA. Retention time of 2,4-D is 21 min and 2,4-D ester is 30 min.

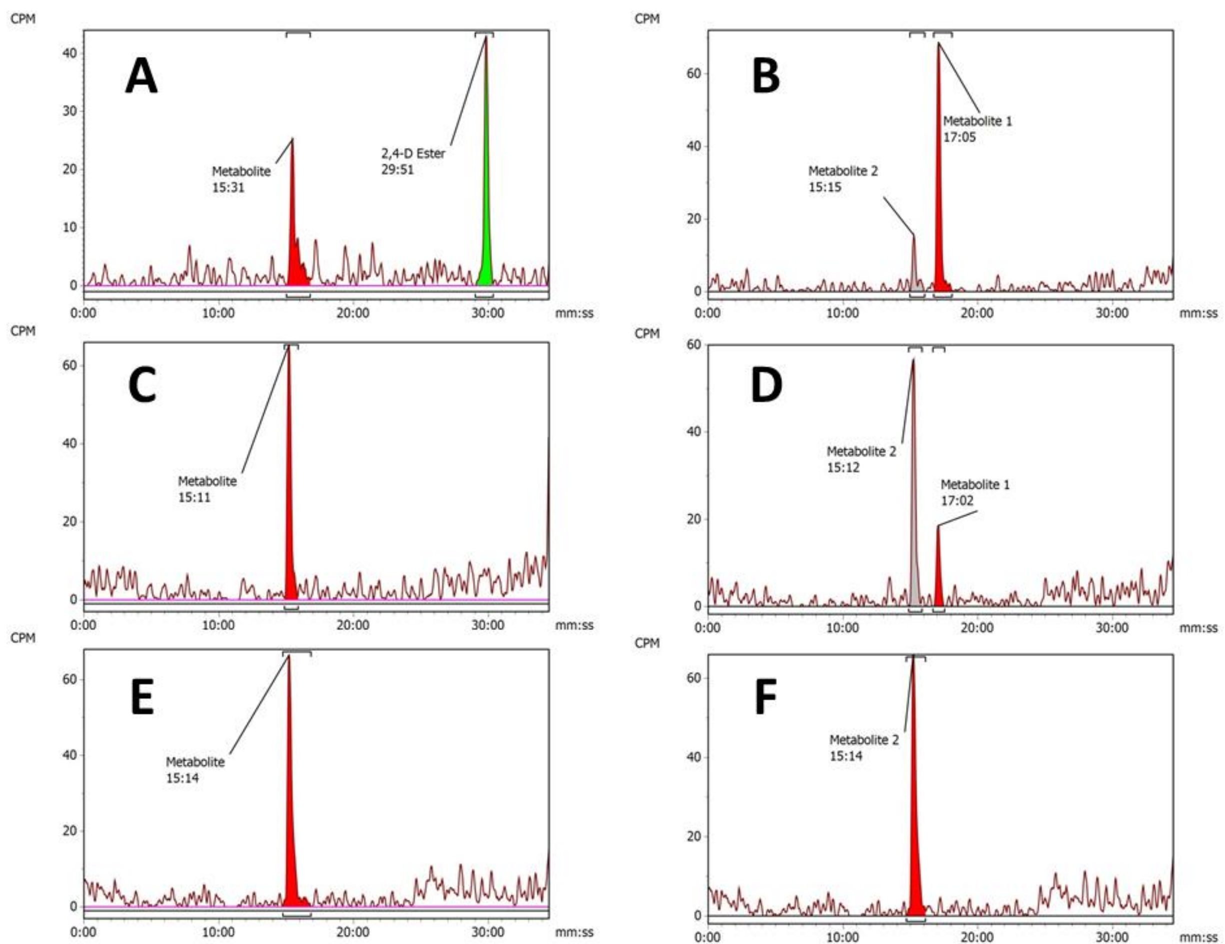


Figure 2.9. HPLC radiochromatograms from the excised-leaf assay quantifying DCP and 2,4-D metabolism in Enlist and NT soybeans at 24 HAA. (A) DCP metabolism by Enlist soybean, (B) DCP metabolism by NT soybean, (C) 2,4-D metabolism by Enlist soybean, and (D) 2,4-D metabolism by NT soybean. Retention time of DCP is 14 min and 2,4-D is 15 min.

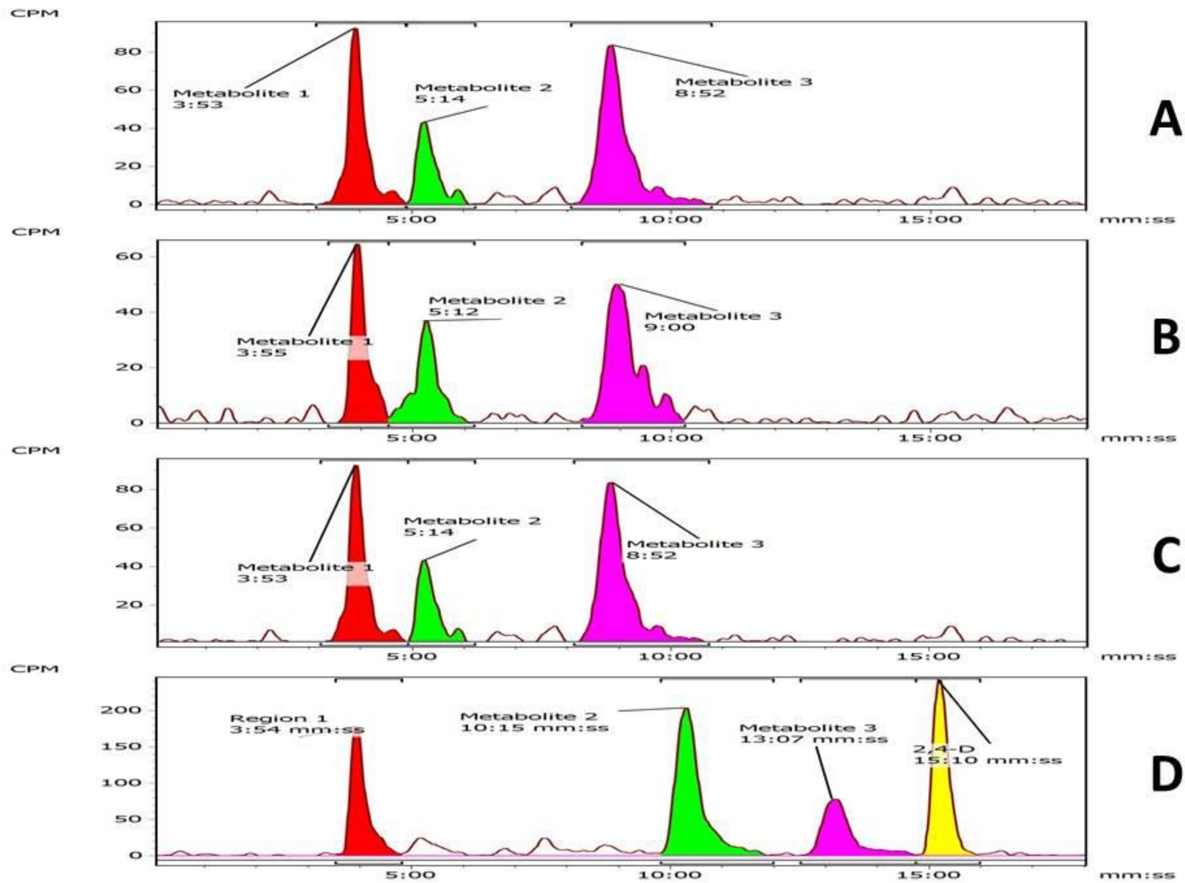
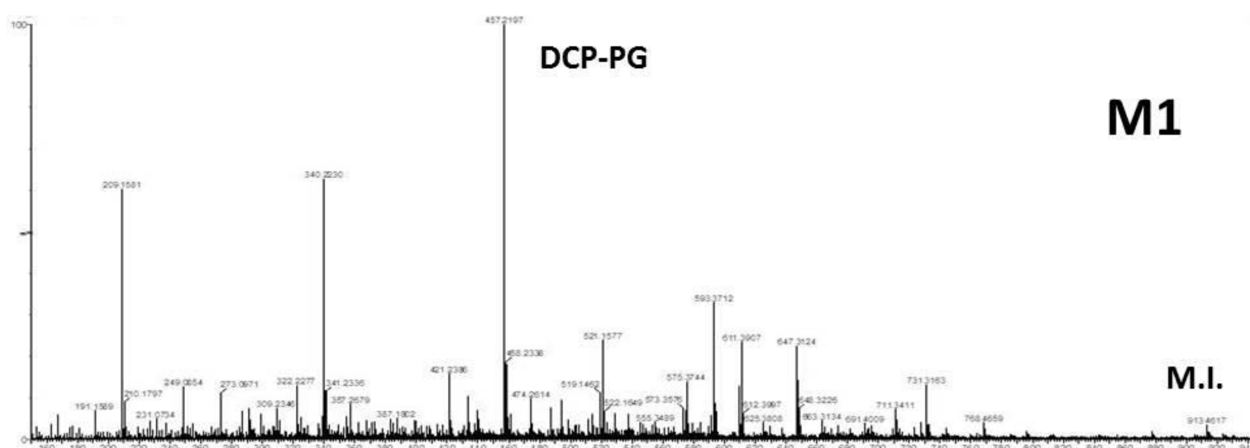


Figure 2.10. Mass spectra of two metabolites derived from 2,4-D metabolism in Enlist soybean. M1 has a molecular ion at a mass-to-charge ratio (*m/z*) of 913 and M2 has a molecular ion of 795 *m/z*. M1 corresponds with the peak at 5 min 52 s from Figure 2.8 and M2 corresponds with the peak at 8 min 12 s. Intermediates previously determined in the metabolism of DCP[27] were detected. The intermediate in M1 (DCP-PG) has a *m/z* of 458 and the intermediate in M2 (DCP-GPG) has a *m/z* of 616.



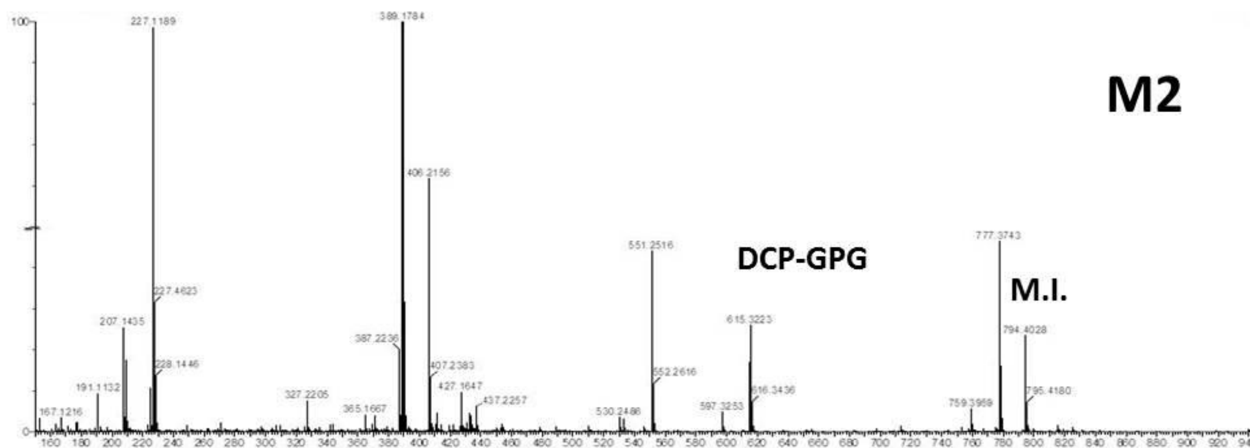
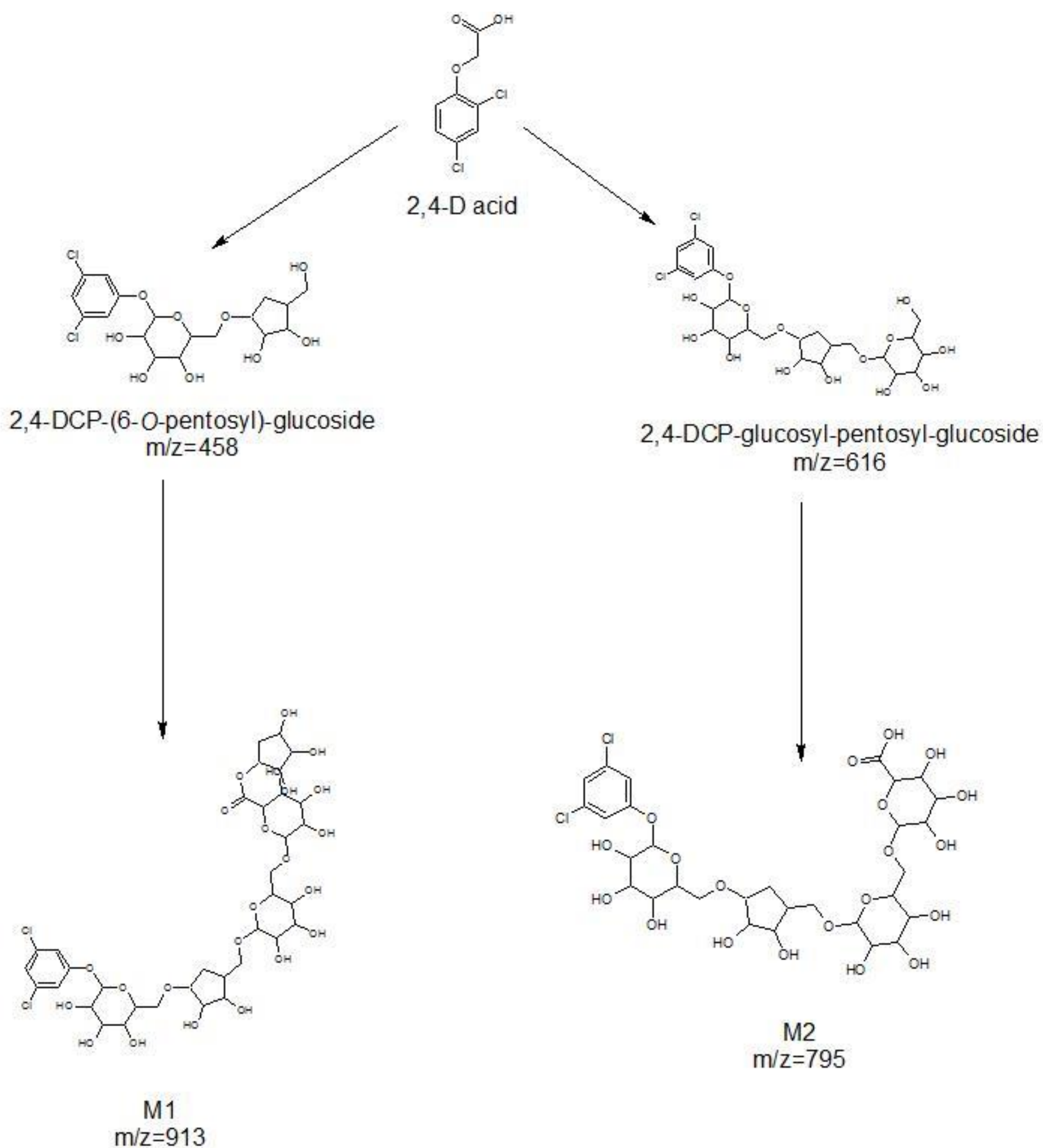



Figure 2.11. Proposed metabolic fate of 2,4-D in Enlist soybean 24 h after herbicide application. LC-MS/MS analysis was conducted on two metabolites and found the formation of two distinct DCP-sugar conjugates. The intermediate metabolites that were previously determined during the metabolism of DCP in previous research[9] and the final M1 and M2 metabolites are shown.



# CHAPTER 3

## UPTAKE, TRANSLOCATION, AND METABOLISM OF 2,4-D IN 2,4-D-RESISTANT CORN (ZEA MAYS)

### Introduction

The Enlist™ Weed Control System provides resistance to 2,4-dichlorophenoxyacetic acid (2,4-D) in several crops including soybean (*Glycine max*), corn (*Zea mays*), and cotton (*Gossypium hirsutum*). Insertion of a transgene belonging to the bacterial enzyme class, aryloxyalkanoate dioxygenase (AAD) confers resistance (Wright et al. 2010). The AAD-1 enzyme cleaves 2,4-D to the nonherbicidal metabolite, dichlorophenol (DCP) *in vitro* (Wright et al. 2010). Injury to Enlist corn is not frequently observed in the field, in contrast, Enlist soybean are occasionally injured following 2,4-D applications but this injury does not affect yield (Robinson et al. 2015). Observing injury in Enlist soybean but not Enlist corn is unusual because the enzyme inserted into Enlist soybean, AAD-12, metabolizes 2,4-D more rapidly than the AAD-1 enzyme inserted in Enlist corn varieties (Wright et al. 2010).

However, unlike soybean, corn is naturally tolerant to 2,4-D, and insertion of the AAD-1 enzyme in Enlist corn provides enhanced 2,4-D tolerance. This enhanced tolerance reduces the risk of auxin-like injury symptoms such as crop leaning, inhibited leaf unfurling, stalk brittleness, deformed brace roots, and reproductive malformations, as well as reduces growth-stage restrictions for postemergence (POST) applications (Wright et al. 2010). The AAD-1 enzyme also provides resistance to the aryloxyphenoxy-propionate (AOPP) family of acetyl-CoA-carboxylase (ACCase) inhibitors, providing Enlist corn with a new site of action for controlling grass weeds (Wright et al. 2010).

The primary difference between the Enlist and non-transformed (NT) corn varieties will be in the metabolic mechanisms in which 2,4-D is detoxified. NT corn can only metabolize 2,4-D through ring-

hydroxylation (Chkanikov et al. 1976 ; Feung et al. 1975; Montgomery et al. 1971; Schroder and Collins 2002) mediated by cytochrome-P450 enzymes (Siminszky 2006), but Enlist corn can also utilize the engineered bacterial pathway that cleaves 2,4-D to produce non-phytotoxic DCP (Wright et al. 2010). Utilizing different metabolic pathways and enzymes will likely result in varying rates of metabolism and unique metabolites formed by the Enlist corn variety, which have not been presently measured.

The objectives of this research were to measure and compare the uptake, translocation, and metabolism of 2,4-D in Enlist and NT corn varieties. This unique research measured these factors in a natural corn variety (NT) compared with a genetically-engineered herbicide-resistant variety (Enlist) and determined how AAD-1 may alter any of these physiological factors. Uptake and translocation of 2,4-D have been measured in corn (Fang and Butts 1954; Hauser 1955; Robertson and Kirkwood 1970; Sargent and Blackman 1972), and 2,4-D metabolism has been determined (Chkanikov et al. 1976; Feung et al. 1975; Montgomery et al. 1971; Schroder and Collins 2002) but not by comparing natural and genetically-engineered 2,4-D resistant varieties.

The impact of glyphosate and the adjuvant from the Enlist Duo formulation (2,4-D choline, glyphosate, and adjuvant) were determined and compared between Enlist and NT varieties. Additionally, comparison of 2,4-D uptake, translocation, and metabolism between corn and soybean (Skelton et al. 2014a) may explain the occurrence of injury in Enlist soybean and lack of injury in Enlist corn. The research hypotheses are: (1) injury to Enlist corn is observed less frequently compared to Enlist soybean due to the combination of natural tolerance and engineered resistance to 2,4-D; (2) uptake levels between corn varieties are equal but may vary between treatments; (3) 2,4-D metabolism in Enlist corn is greater than NT, which may result in translocation differences; (4) the metabolic fate of 2,4-D is quantitatively different between Enlist and NT corn.

## Materials and Methods

### *Whole-Plant Assay*

Enlist and NT corn varieties (provided by Dow AgroSciences, Indianapolis, IN 46268) were grown to the V2 growth stage in a growth chamber (16 hour day/8 hour night, 28˚C/26˚C, and photosynthetically active radiation was 490 µmol/$m^2$s). The Enlist and NT varieties are isogenic lines that share the same genetic background with the only difference being the 2,4-D-resistance trait. The V2 growth stage was selected to be consistent with previous research (Skelton et al. 2013, 2014a). The first experiment focused on measuring 2,4-D uptake with various treatments containing 2,4-D, glyphosate, and the adjuvant from Enlist Duo (Adj.), and the second experiment focused on a situation when injury is induced in Enlist corn (Table 3.1).

At V2, the plants were treated with herbicide treatments 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 applying the radiolabeled herbicide treatments. Twenty µM [Uniformly Ring-Labeled (URL)-$^{14}$C]-2,4-D radiolabeled solutions (specific activity 1.4 GBq/mmol) were the same as the whole-plant spray solutions but spiked with radiolabeled 2,4-D (Table 3.1). Herbicide solutions were applied using a glass syringe (Hamilton Co., Reno, NV 89502) to deliver 10 µL (100 Bq) divided into 33 droplets to the treated leaf. The second leaf was used as the treated leaf and was noted with a marker for clear recognition during harvesting. Treatments without an adjuvant required the treated leaf being fixed horizontally and applied with one-10 µL droplet (100 Bq) to prevent the droplet from running off the leaf surface. This method maintained similar uptake levels when treatments with the adjuvant were used as a comparison (data not shown).

After the radiolabeled solution was applied, the plants were returned to the growth chamber until sample collection was conducted at 1, 3, 6, 12, and 24 h after application (HAA). At sampling, the plant was divided into three parts: the treated leaf, above the treated leaf portion, and below the treated leaf portion. The treated leaf was the entire leaf to which the radiolabeled herbicide solution was applied. The above and below the treated leaf portions were determined by dividing the plant into two halves based upon the collar of the treated leaf. The treated leaf was immediately rinsed in 3 mL of a water:methanol solution (80:20, v/v) for 45 s to remove any remaining $^{14}$C-2,4-D material from the leaf surface in a scintillation vial. The methanol rinse solution was then analyzed via liquid scintillation spectrometry (LSC, Model 1900 TR, Packard Instrument, Meriden, CT 06450) to determine the amount of $^{14}$C-2,4-D left on the surface. The treated leaf, above, and below the treated leaf portions were allowed to air dry before the portions were combusted for 4 min in a biological oxidizer (Model OX-500, R.J. Harvey Instrument Corp., Hillsdale, NJ 07462) and analyzed via LSC to show amount of $^{14}$C-labeled material within the plant portion. Total radioactive material recovery was determined by summing the amount of $^{14}$C-material recovered from each step and averaged 90% for all experiments.

Enlist and NT corn plants were analyzed with photostimulated luminescence (PSL) to qualitatively determine where $^{14}$C-material is located in the plant at 24 HAA. Enlist and NT plants were treated in a similar fashion as in the whole-plant assays, but a higher amount of $^{14}$C-2,4-D was used (416 Bq). After air drying, samples were placed on a phosphorimage exposure cassette (Molecular Dynamics) for 48 h before analyzing by PSL (Typhoon 9400 Variable Mode Imager, Amersham Biosciences, Buckinghamshire, England). Dried samples were oxidized to determine the amount of $^{14}$C-material (as percent absorbed) in each portion as a reference to the phosphorimage.

*Excised-Leaf Assays*

***Metabolism of 2,4-D in Enlist 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 in the plant and produce more accurate metabolism-degradation results. The method used for the excised- leaf assay utilized similar methods as in research with a dicot weed, *Amaranthus tuberculatus* (Ma et al. 2013, 2015). Both varieties were grown in a growth chamber to the V2 growth stage to be consistent with the whole-plant assay, and the second leaf was used to measure 2,4-D metabolism. The treated leaf was cut from the stem at the collar, and then placed under water and cut again to ensure that air was not trapped in the vascular tissue. The excised leaf assay was divided into three stages: (1) 0.1 M Tris buffer pH 6.5, (2) herbicide solution, and (3) water. After removing the leaf from the plant, the leaf was inserted into a 1.5 mL Eppendorf tube containing 200 µL of the buffer solution for 0.5 h to acclimate to the buffer. The leaf was then transferred to a new Eppendorf tube containing 100 µL (4.2 kBq) of the herbicide solution for 1 h. The herbicide solution was 25 µM $^{14}C$-2,4-D (specific activity 1.4 GBq/mmol) in 0.1 M Tris buffer pH 6.5. The treatments were 2,4-D only and 2,4-D + glyphosate (35.5 mM). After 1 h, almost all of the herbicide solution had been absorbed and the remaining amount was quantified using LSC. Finally, the leaves were transferred to an Eppendorf tube containing 1.5 mL of distilled water and would remain in the tubes until sample collection at 1, 3, 6, 12, and 24 HAA. Distilled water was added throughout this stage to ensure that the leaf had a constant supply of water and was not stressed. Distilled water was used instead of half-strength MS salt solution (Ma et al. 2013, 2015) because injury developed when MS salt solution was used with this assay in soybean.

At each sampling time, the leaf was removed from the solution and processed to extract the $^{14}C$-material. The leaf was frozen with liquid nitrogen, homogenized with a glass rod, and treated with 14

mL of a water:acetone solution (10:90, v/v) for 16 h at -4˚C. Following acetone extraction, samples were centrifuged at 12000*g* for 10 min resulting in two distinct phases, a solid (non-extractable) and liquid (extractable) portion. The non-extractable portion was allowed to air dry before oxidization to determine the amount of $^{14}$C-material as bound residue. The liquid portion was analyzed using reverse-phase high pressure liquid chromatography (RP-HPLC) to determine the metabolism of $^{14}$C-2,4-D. Samples for HPLC analysis were generated by concentration at 40˚C with a rotary evaporator (Rotavapor R-200, BÜCHI, Flawil, Switzerland 9230) 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 LSC. Reverse phase HPLC samples were normalized so 38.5 Bq was injected into HPLC. Recovery of $^{14}$C-material ranged from 92 to 98%.

Reverse-phase HPLC was performed on a Perkin Elmer Flexar LC HPLC (Model N2910401, Perkin Elmer, Akron, OH 44311) with an Altima $C_{18}$ column (4.6 X 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 (25 min total). Radiolabeled compounds were detected with a β-RAM Radio-HPLC Detector (Model 4, Lab Logic, Brandon, FL 33511) and Ultima-Flo M cocktail (Perkin Elmer). [Uniformly Ring-Labeled (URL)-$^{14}$C]-2,4-D displayed a retention time of 21 min. Figure 3.1 shows a typical chromatogram of Enlist and NT corn 2,4-D metabolism at 1 and 24 HAA.

***DCP metabolism in Enlist and NT corn.*** The excised-leaf assay was used to determine the metabolism of DCP. DCP was utilized because it is the first metabolite formed in Enlist corn by the AAD-1 enzyme (Wright et al. 2010) and would show if DCP is metabolized in a similar fashion between corn varieties

regardless of the transgene, as determined in 2,4-D-resistant and sensitive cotton (Laurent et al. 2006). An assay utilized 25 µM $^{14}$C-DCP (specific activity 1.2 GBq/mmol) to measure DCP metabolism at 1 and 24 HAA. Samples generated using excised-leaf assays were analyzed by HPLC as previously described to determine the metabolite(s) formed in Enlist and NT corn but with modifications outlined below.

Reverse-phase HPLC was performed using the same system as the previous excised-leaf assays, but utilized a different column and mobile phase. A ThermoScientific $C_4$ Column (Hypersil Gold C4, 4.6 x 250 mm, 5 µm) was used to analyze samples. Eluent A was 0.1% (v/v) formic acid in water and eluent B was acetonitrile. The 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 14 min.

*Statistical Methods*

Treatments were arranged in a completely randomized design and data from each independent experiment was combined and analyzed. All experiments were replicated twice and contained three biological replications for each treatment combination. Uptake and metabolism 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).

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

In the model, $Y$ represents the percentage of 2,4-D uptake or metabolism at time $t$, $\alpha$ is the parameter that estimates the maximum amount of $Y$, and $\beta$ is the parameter that estimates the $t$ to reach 50% $\alpha$. Parameters of different models were compared using pairwise differences ($\alpha$=0.05) with the *compParm*

function in R. The translocation and non-extractable $^{14}C$-material data were analyzed with SAS (SAS version 9.4, SAS Institute Inc., Cary, NC 27513) using Proc Mixed in a split-plot design with the whole-plot being each time point and the sub-plot being the treatment combinations. Means were separated using Tukey's Honest Significant Difference test at α=0.05. Experiment by treatment interactions were not detected, so results were pooled over both experimental replications for all data.

**Results and Discussion**

*Whole-Plant Assays*

***Uptake of 2,4-D in Enlist and NT corn.*** The amount ($U_{max}$; P=0.2549) and rate ($U_{50}$; P=0.4338) of 2,4-D uptake measured were not different between the Enlist and NT corn varieties. In contrast, a significant difference between the $U_{max}$ (P=0.0210) among the treatments was discovered but not the $U_{50}$ (P=0.1891; Table 3.2). The range in rate of 2,4-D uptake was wide with the lowest $U_{50}$ value at 2.5 h with the 2,4-D + adjuvant treatment and the greatest at 5.1 h with the Enlist Duo treatment (Table 3.2). The dramatic increase in 2,4-D uptake derived from the Adj. measured in soybean (Skelton et al. 2013, 2014a) was not measured in corn. The only significant difference in maximum uptake was between the 2,4-D + Adj. and Enlist Duo treatments, and the treatments without the Adj. had similar uptake levels as treatments with the Adj. (Table 3.2). At 1 HAA, all treatments had equivalent 2,4-D uptake levels, but from 3 to 12 HAA the Enlist Duo treatment had significantly lower uptake levels than all other treatments (Fig. 3.2). By 24 HAA, Enlist Duo (69%) had significantly lower 2,4-D uptake levels than 2,4-D + Adj. (89%) only (Fig. 3.2).

The translocation of $^{14}C$-material was not significantly different (P=0.2664) between varieties. The amount of $^{14}C$-material remaining in the treated leaf was equivalent for both varieties (Table 3.3). There was a significant difference (P=<0.0001) between the above and below the treated leaf portions in

the limited amount of radiolabeled material that translocated from the treated leaf (P=<0.0001). The movement of $^{14}$C-material was more predominant to the above portion (1.9% $^{14}$C-material absorbed) compared with the below portion (0.7%). At 1 and 3 HAA, the amount of radiolabeled material was equal between the two parts, but from 6 HAA and on, the amount in the above portion was greater than the below portion. Figure 3.3 shows a phosphorimage of Enlist and NT corn plants at 24 HAA. The location of the $^{14}$C-material is heavily concentrated in the treated leaves (Fig. 3.3 A and B; 83% and 94%), but a portion of the $^{14}$C-material is located in the rest of the plant (Fig. 3.3 C and D; 17% and 6%), mainly in the stem around the point of attachment of the treated leaf.

***Injuring Enlist Corn and the Effect on 2,4-D Uptake.*** Injury to Enlist corn was not frequently observed during field studies as with Enlist soybean, and 2,4-D uptake levels were lower in corn compared with soybean (Skelton et al. 2013, 2014a). When Enlist Duo was tank-mixed with formulated acetochlor (Table 3.1), injury developed that resembled the injury witnessed in Enlist soybean (Fig. 3.4). A whole-plant assay was conducted to test the hypothesis that injury to Enlist corn results from increased 2,4-D uptake when Enlist Duo and acetochlor are tank-mixed. There was a significant difference in 2,4-D uptake between the two treatments (P=<0.0001). When Enlist Duo was applied without acetochlor, 2,4-D uptake was 30%, but 2,4-D uptake increased to 42% overall with acetochlor.

The adjuvant from Enlist Duo did not increase the uptake of 2,4-D in corn, and glyphosate did not influence 2,4-D uptake (Table 3.2). The Enlist Duo treatment resulted in the least amount of 2,4-D uptake in corn, which is in contrast to what was measured in soybean (Skelton 2013, 2014a). The lack of an increase in 2,4-D uptake from the Enlist Duo adjuvant may be related to the adjuvant formulation being targeted to control dicot weeds instead of grasses since 2,4-D does not control grasses. In support of this theory, the lack of an adjuvant improving herbicide uptake has been measured in other plants with various adjuvants and adjuvant physiochemical characteristics (Wang and Liu 2007). Uptake of 2,4-

D significantly increased and injury developed in Enlist corn when Enlist Duo was tank mixed with a chloroacetamide herbicide (Fig. 3.4). Elevated concentrations of 2,4-D derived from increased 2,4-D uptake levels may contribute to the injury in Enlist corn. Since the enzyme inserted in Enlist corn, AAD-1, metabolizes 2,4-D at a slower rate than the enzyme in Enlist soybean, AAD-12 (Wright et al. 2010), Enlist corn may be more sensitive to increased uptake levels triggered by certain tank-mix combinations. Enlist Duo plus formulated acetochlor resulted in an increase in 2,4-D uptake from 30% to 42%, which is much lower than the 2,4-D levels that induced injury in Enlist soybean (Skelton et al. 2013, 2014a). AAD-1 is more prone to being overwhelmed by elevated cellular 2,4-D concentrations compared to AAD-12 due to its slower rate of 2,4-D metabolism. This may be a potential factor in understanding why injury develops at a relatively small increase in 2,4-D uptake in Enlist corn compared with Enlist soybean (Skelton et al. 2013, 2014a).

Differences in translocation were not detected between isogenic lines (varieties). The natural tolerance of corn to 2,4-D may explain the lack of difference in translocation between Enlist and NT corn, in contrast to Enlist and NT soybean (Skelton et al. 2013, 2014a). Translocation of 2,4-D acid away from the treated leaf via the phloem is likely precluded in both corn varieties by the rapid detoxification of 2,4-D and sequestration of polar metabolites (Chkanikov et al. 1976; Feung et al. 1975; Montgomery et al. 1971; Robertson and Kirkwood 1970; Schroder and Collins 2002). However, non-toxic metabolites may form slower in NT corn than in Enlist corn, so methods developed for metabolism studies with excised leaves (Kreuz and Fonne-Pfister, 1992; Ma et al. 2015) were utilized to determine differences in metabolite formation between corn varieties.

*Excised-Leaf Assays*

***Metabolism of 2,4-D in Enlist and NT Corn.*** A significant difference was determined in the amount ($M_{max}$; P=0.0051) of 2,4-D metabolized between the two corn varieties but not when measuring rates ($M_{50}$; P=0.6174; Table 3.4). Enlist corn required 2.3 h compared to 2.0 h for NT corn to metabolize 50% of 2,4-D absorbed. The $M_{max}$ of Enlist corn was greater (100%) compared to NT corn (83.9%). Metabolism of 2,4-D was equal at 1 and 3 HAA for both varieties, but the Enlist variety had 11 to 13% more 2,4-D metabolized at each successive time point (Fig. 3.5). The addition of glyphosate did not significantly affect the $M_{max}$ (P=0.1143) or $M_{50}$ (P=0.0668; Table 3.4).

The amount of non-extractable $^{14}C$-material between the two varieties was not different (P=0.7548). Enlist corn had 40% non-extractable material compared to 41% in the NT variety. There was a significant difference between treatments (P=<0.0001); plants treated with 2,4-D + glyphosate (47%) accumulated more non-extractable material compared to the 2,4-D only treatment (34%). This can be explained through the significant variety by treatment by hour interaction (P=0.0018). NT corn treated with 2,4-D only had lower non-extractable material at all time points except for 24 HAA compared to NT corn treated with 2,4-D + glyphosate and both treatments in the Enlist variety. A difference between 2,4-D only or 2,4-D + glyphosate in non-extractable material in Enlist corn was not measured at any time point.

***DCP Metabolism in Enlist and NT Corn.*** Metabolism of DCP in Enlist and NT corn was similar, and DCP was not detected as a metabolite at any time point in either variety (Fig. 3.6 A and B). This implies that corn rapidly metabolizes DCP and does not accumulate during the time course study. By 24 HAA, both varieties formed four distinct metabolites (Fig. 3.6 A and B; retention times are approximately 4 min, 5 min, 6.5 min, and 11 min), but only one metabolite (11 min) was specific to DCP metabolism. The rapid

metabolism of DCP matches results from previous studies in wheat (*Triticum aestivum*), soybean, tobacco, and several other edible dicot plants (Laurent et al. 2006, 2007; Pascal-Lorber et al. 2003). This indicates that when applied as a non-herbicidal substrate, Enlist and NT corn varieties metabolize DCP similarly, and the endogenous enzymes in corn metabolize DCP.

Enlist corn treated with 2,4-D formed the metabolite specific to DCP metabolism (Fig. 3.6 A and C; approximately 11 min). Since 2,4-D is being rapidly converted to DCP by the AAD-1 enzyme in Enlist corn and then further metabolized, polar metabolite(s) of DCP are found when supplying excised Enlist corn leaves with 2,4-D (Fig. 3.6 A and C). However, the peak at approximately 11 min is not detected when NT corn leaves are supplied with 2,4-D (Fig. 3.6 D). Three metabolites (approximately 4 min, 5 min, and 6.5 min) and a peak specific to NT corn (Fig. 3.6 D; approximately 24 min) were detected in Enlist and NT corn treated with 2,4-D (Fig. 3.6 C and D). These peaks may contain ring-hydroxylated metabolites generated by the natural 2,4-D metabolic pathway characterized by previous studies with corn, wheat, soybean, tobacco, and several other dicot and monocot species (Chkanikov et al. 1976; Feung et al. 1975; Montgomery et al. 1971; Robertson and Kirkwood 1970; Schroder and Collins 2002).

Enlist corn metabolized a greater amount of 2,4-D than NT corn, and glyphosate did not alter 2,4-D metabolism in either variety (Table 3.4). The excised-leaf assay provided new metabolic information to support previous field studies showing that Enlist corn can tolerate higher rates of 2,4-D (Wright et al. 2010). Non-extractable radiolabeled material accumulation was not different between varieties. This finding correlates with the equivalent amount of translocation between corn varieties determined by the whole-plant assay (Table 3.3). Rapid detoxification of 2,4-D to immobile, polar metabolites may inhibit translocation to meristematic tissues of both varieties, as well as storage or sequestration of metabolite(s) in the treated leaves due to Phase III detoxification mechanisms (Van Eerd et al 2003; Hatzios 2005). The metabolites formed during 2,4-D metabolism by Enlist and NT corn

varieties (Fig. 3.6 C and D) are different due to the conversion of 2,4-D to DCP by AAD-1 in Enlist corn. Dichlorophenol is further metabolized to a unique metabolite (Fig. 3.6 C; approximately 11 min) in Enlist corn that is not present when 2,4-D is used as a substrate in NT corn (Fig. 3.6 D).

Injury to Enlist soybean may occur when 2,4-D, glyphosate, and the adjuvant from Enlist Duo are used either as the premix product, Enlist Duo, or as a tank mix (Skelton et al. 2013, 2014a). Glyphosate does not alter 2,4-D uptake or metabolism in corn, and Enlist Duo results in relatively slower uptake of 2,4-D compared with previous findings in Enlist soybean (Skelton et al. 2013, 2014a). As a result, the combination of the lower amount and rate of 2,4-D uptake in conjunction with endogenous detoxification mechanisms of 2,4-D reduces the likelihood of injury in corn by reducing the potential for overwhelming the metabolic capacity of the AAD-1 enzyme (Skelton et al. 2013, 2014a). However, injury was observed in Enlist corn when 2,4-D uptake levels were significantly increased following a tank mix application of Enlist Duo with formulated acetochlor, but this research did not determine which component(s) of formulated acetochlor contributed to this increase in uptake. For example, the active ingredient (acetochlor), formulation, organic solvent, or a combination of these components may have contributed to the increase in 2,4-D uptake. Enlist corn may be more sensitive to excessive cellular 2,4-D concentrations since AAD-1 and the endogenous detoxification enzymes in corn combined metabolize 2,4-D less rapidly than the AAD-12 enzyme in Enlist soybean (Skelton et al. 2014b). Alternatively, a component of the formulated acetochlor and/or the interaction of the acetochlor product with 2,4-D may have induced injury instead of the increase in 2,4-D uptake. Further research should focus on determining the cause of injury with this tank mixture under greenhouse and field conditions.

Insertion of the *aad-1* transgene in Enlist corn confers enhanced 2,4-D tolerance and AOPP-resistance (Wright et al. 2010). These novel traits increase the number of 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). However, the potential for development of additional 2,4-D-resistant weed species and populations is a risk of using a continuous rotation of Enlist crops because the use of 2,4-D multiple times a year, and potentially every year, is possible compared to current practices in which 2,4-D cannot be used in soybean. The anticipated increase in selection pressure for 2,4-D-resistant weed populations (Mortensen et al. 2012) warrants further investigations into the rate of development and spread of 2,4-D-resistant weeds under field conditions; use of integrated management strategies to prevent resistant weed development; how to effectively manage existing 2,4-D-resistant weeds; and understanding basic physiological and genetic mechanisms that confer 2,4-D resistance.

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**Tables**

Table 3.1. Treatment list for whole-plant assays used to determine 2,4-D uptake and translocation in Enlist and non-transformed corn varieties.

| Experiment 1 | Experiment 2 |
|---|---|
| 2,4-D choline only[a] | Enlist Duo |
| 2,4-D + Adj.[b] | Enlist Duo + Surpass NXT[e] |
| 2,4-D + Glyphosate[c] | |
| 2,4-D + Glyphosate + Adj. | |
| Enlist Duo™[d] | |

[a] 2,4-D choline (1065 g ae/ha, 2,4-D choline 3.8L, Dow AgroSciences, Indianapolis, IN 46268).

[b] Adjuvant (Enlist Duo Adjuvant Mix, Dow Agrosciences, Indianapolis, IN 46268).

[c] Glyphosate (1120 g ae/ha, Glyphosate dimethylammonium salt 781.254 g $L^{-1}$, Dow AgroSciences, Indianapolis, IN 46268).

[d] Enlist Duo (2185 g ae/ha, Enlist Duo 400 g ae $L^{-1}$, Dow AgroSciences, Indianapolis, IN 46268).

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

AMS (2.5% v/v, N-PAK AMS Liquid 34%, Dow AgroSciences, Indianapolis, IN 46268) was added to all treatments.

Table 3.2. Nonlinear regression parameters for 2,4-D uptake from whole-plant assays with Enlist and non-transformed corn varieties.

| Treatment | $U_{max}$ [a,b] | $U_{50}$ [c,d] |
|---|---|---|
| 2,4-D only | 86.5 ab | 3.4 |
| 2,4-D + Adjuvant | 101.2 a | 2.5 |
| 2,4-D + Glyphosate | 90.4 ab | 3.9 |
| 2,4-D + Glyphosate + Adjuvant | 87.6 ab | 2.6 |
| Enlist Duo | 75.6 b | 5.1 |

[a] Parameter corresponding to the modelled maximum 2,4-D uptake (% recovery) of treatment.

[b] Treatments followed by the same letter within each experiment are not significantly different by Fisher's LSD Test $\alpha=0.05$.

[c] Parameter corresponding to time (hours) for treatment to reach 50% $U_{max}$.

[d] $U_{50}$ values for treatments not statistically different ($P=0.1891$).

Table 3.3. Distribution of $^{14}C$-material from whole-plant assays using Enlist and non-transformed and corn varieties.

| Variety | Portion | Hours After Application | | | |
|---|---|---|---|---|---|
| | | 1 | 3 | 6 | 24 |
| | | % $^{14}C$-Material in Plant[a,b] | | | |
| Non-Transformed | Treated Leaf | 97.3 | 100.0 | 96.0 | 92.6 |
| | Above | 0.7 | 0.4 | 1.8 | 3.8 |
| | Below | 0.4 | 0.3 | 0.6 | 2.3 |
| Enlist | Treated Leaf | 99.3 | 100.0 | 95.9 | 95.0 |
| | Above | 1.3 | 1.0 | 1.6 | 3.8 |
| | Below | 0.2 | 0.1 | 0.4 | 1.5 |

[a] No significant difference between corn varieties in amount $^{14}C$-material remaining in treated leaf (P=0.2664).

[b] No significant interaction between variety, part, and hour (P=0.7462).

Table 3.4. Nonlinear regression parameters for 2,4-D metabolism from the excised-leaf assays with Enlist and non-transformed (NT) and corn varieties.

| | | $M_{max}$ [a] | $M_{50}$ [b] |
|---|---|---|---|
| Variety | Treatment | % Metabolized[c,d] | Hours[e] |
| Enlist | | 100.0 a | 2.3 |
| NT | | 83.8 b | 2.0 |
| | 2,4-D Only | 97.4 | 2.7 |
| | 2,4-D + Glyphosate | 87.1 | 1.8 |

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

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

[c] Treatments followed by the same letter within each experiment are not significantly different by Fisher's LSD Test α=0.05.

[d] $M_{max}$ for variety significantly different (P=0.0051) and treatment not significantly different (P=0.0932).

[e] $M_{50}$ for corn variety and treatments not significantly different (P=0.6174, 0.1143).

**Figures**

Figure 3.1. Representative HPLC radiochromatograms from the excised-leaf assay to determine the amount of parent $^{14}C$-2,4-D (retention time 21 min) at each time point. (A) Enlist corn at 1 HAA, (B) NT corn at 1 HAA, (C) Enlist corn at 24 HAA, and (D) NT corn at 24 HAA.

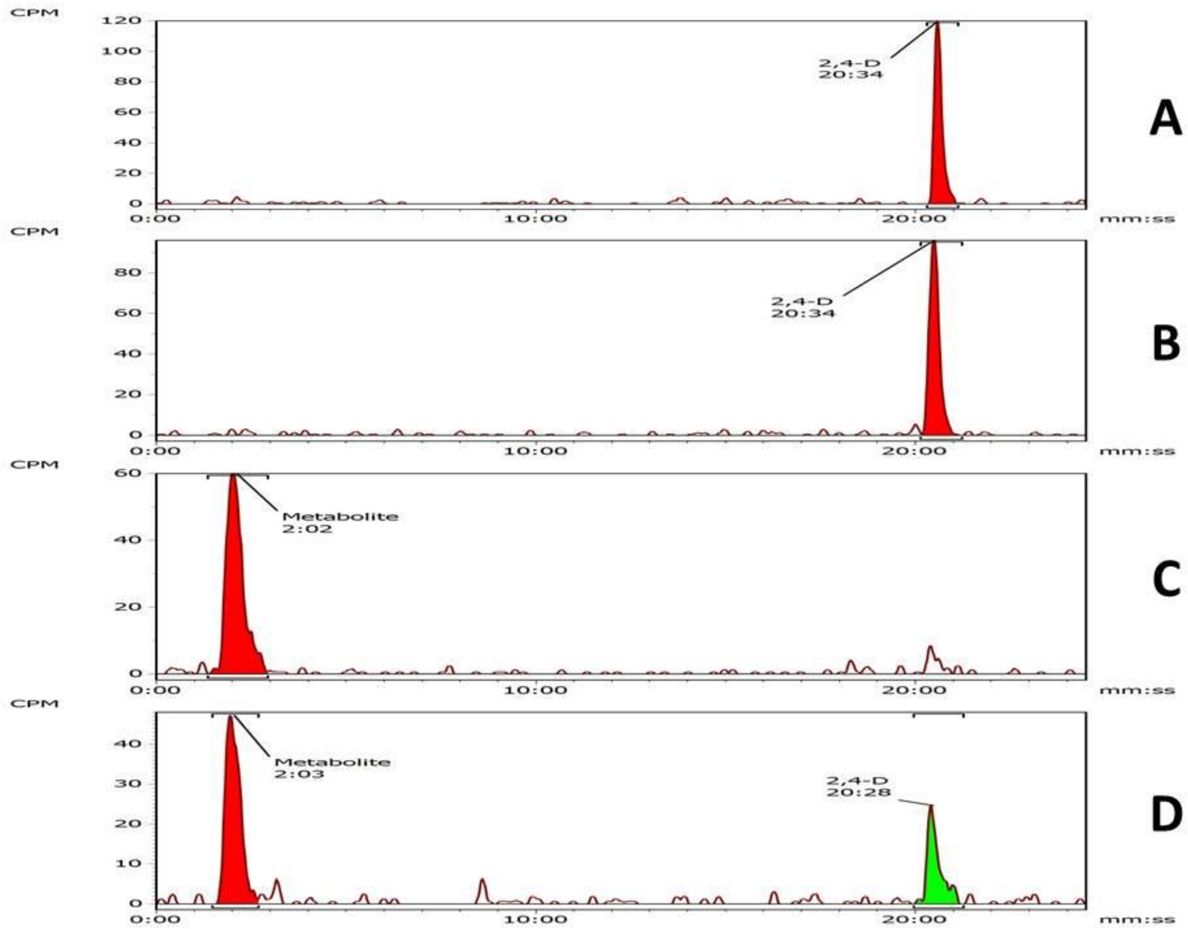


Figure 3.2. Uptake of 2,4-D among the five treatments from the whole-plant assay with Enlist and non-transformed corn during the 24 h time course. Markers represent the average uptake among treatments (1) 2,4-D only, (2) 2,4-D + Glyphosate, (3) 2,4-D + Adjuvant from Enlist Duo (Adj.), (4) 2,4-D + Glyphosate + Adj., and (5) Enlist Duo. All treatments were applied at the same rate of 2,4-D choline (1065 g ae/ha), glyphosate (1120 g ae/ha), adjuvant, and AMS (2.5% v/v) was included.

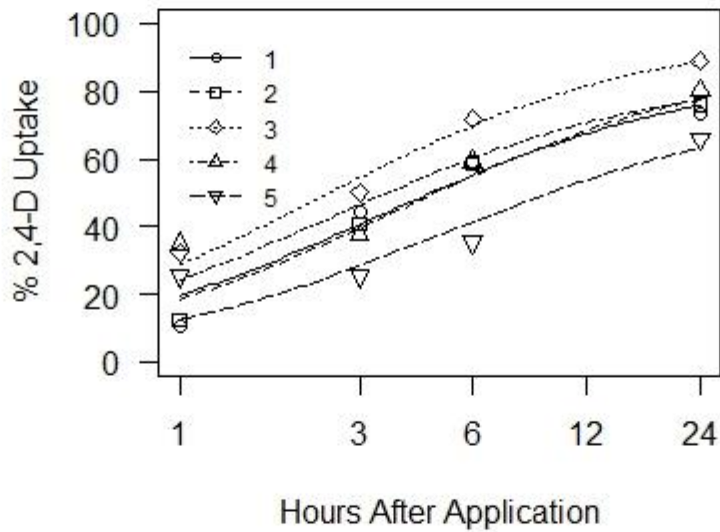


Figure 3.3. Phosphorimage and picture of Enlist and non-transformed (NT) corn at 24 HAA treated with $^{14}C$-2,4-D. Areas of increased intensity indicate a relatively higher amount of radiolabeled material. (A) NT treated leaf, (B) Enlist treated leaf, (C) NT above and below treated leaf portion, and (D) Enlist above and below treated leaf portion. Both Enlist and NT roots were analyzed but were not visible on the phosphorimage due to amounts of $^{14}C$-material below the detection threshold. Arrows indicate 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: (A) 83%, (B) 94%, (C) 17%, and (D) 6%.

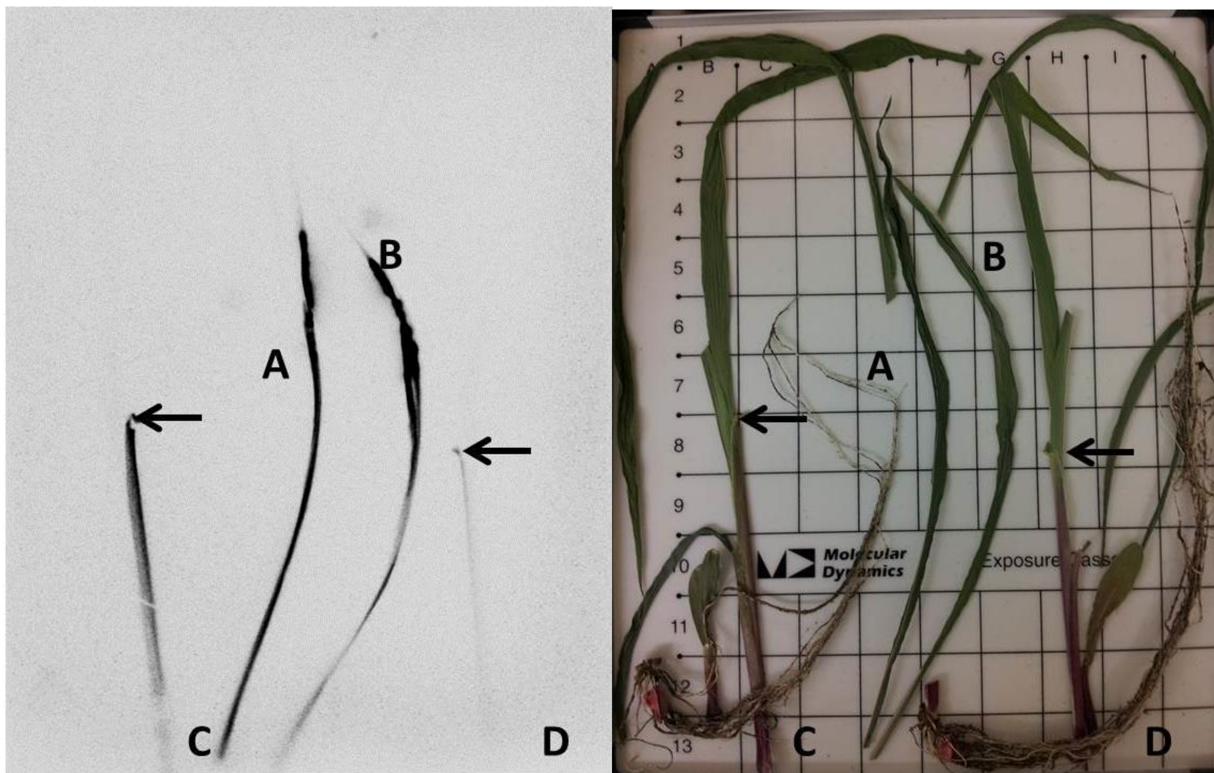


Figure 3.4. Enlist corn plants 7 d after an herbicide application with (A) Enlist Duo, (B) Enlist Duo + acetochlor, and (C) acetochlor. The tank-mixture of Enlist Duo and acetochlor (B) injured Enlist corn (arrows), but applications of Enlist Duo (A) or acetochlor (C) did not result in injury. Uptake of 2,4-D increased from 30% with Enlist Duo alone to 42% when acetochlor was included.

Figure 3.5. Metabolism of 2,4-D in Enlist (E) and non-transformed (NT) corn varieties from the excised-leaf assay. Markers represent the average amount of 2,4-D metabolized and error bars represent standard error of the non-linear regression model at each time point.

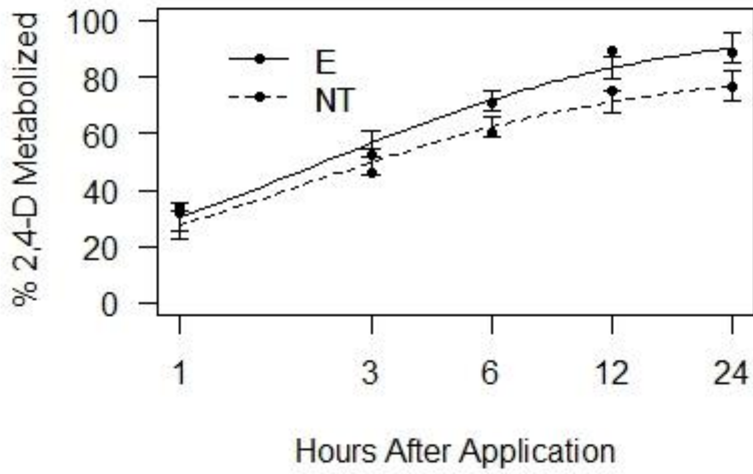
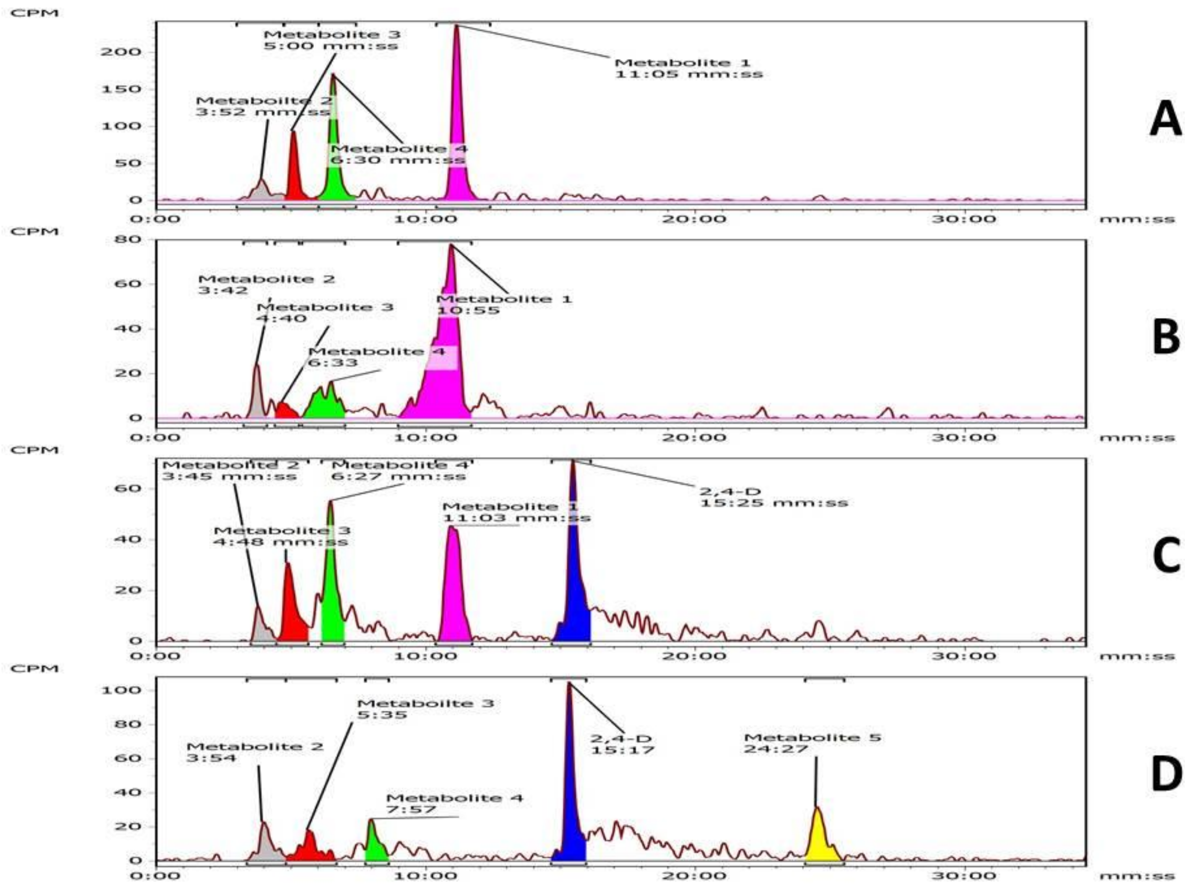


Figure 3.6. HPLC radiochromatograms determining DCP and 2,4-D metabolism in Enlist and non-transformed (NT) corn excised-leaves at 24 HAA. (A) DCP metabolism by Enlist corn, (B) DCP metabolism by NT corn, (C) 2,4-D metabolism by Enlist corn, and (D) 2,4-D metabolism by NT corn. Retention time of DCP is 14 min and 2,4-D is 15 min.



## CHAPTER 4

## WATERHEMP CONTROL UNDER DROUGHT STRESS WITH 2,4-D AND GLYPHOSATE

### Introduction

Waterhemp (WH, *Amaranthus tuberculatus*) is a difficult to control annual weed species in cropping systems throughout the United States. Waterhemp is a dioecious species capable of producing up to one million seeds per female plant (Steckel 2007). The competitive nature, extended seed germination and emergence (Hartzler et al. 1999), obligate outcrossing reproductive biology, and rapid development of herbicide-resistant biotypes (Steckel 2007) make WH an important weed to manage in agricultural cropping systems.

The need to control weeds experiencing abiotic stresses in arable areas will be essential for continued food and fiber production (Ali and Talukder 2008; Passioura 2006) with the potential for longer periods without rainfall or drought (Burke and Brown 2008; Rind et al. 1990). The moderate loss of water in the plant when transpirational water loss exceeds root absorption creates drought stress (Jaleel et al 2009; Patterson 1995). Symptoms of drought stress include reduced water content, stomatal closure, loss of turgor, and decreased cell enlargement and growth (Jaleel et al 2009). Prolonged periods of drought stress results in the reduction of photosynthesis, disturbance of metabolism, and plant death (Jaleel et al 2009). The chemical control of weeds under drought stress is more difficult compared to unstressed plants (Adkins et al. 1998a, 1998b; Boydston 1990; Dickson et al. 1990; Patterson 1995; Tanpipat et al. 1997; Wicks and Hanson 1995; Zhou et al. 2007). Drought stress influences herbicide uptake, translocation, metabolism, and efficacy (Patterson 1995). Plants grown under drought conditions can develop thicker cuticles or leaf pubescence, which inhibit herbicide

uptake, and lowered levels of photosynthesis and photoassimilate transport contribute to reduced herbicide translocation (de Ruiter and Meinen 1998; Patterson 1995).

Glyphosate and 2,4-dichlorophenoxyacetic acid (2,4-D) are two systemic postemergence (POST) herbicides commonly used in cropping systems to control WH. The effect of drought stress on glyphosate efficacy has be extensively studied (Adkins et al. 1998a, 1998b; Boydston 1990; Dickson et al. 1990; Patterson 1995; Tanpipat et al. 1997; Wicks and Hanson 1995; Zhou et al. 2007), but less is known about drought stress and 2,4-D efficacy and neither herbicide has been studied for controlling drought-stressed WH. The objectives of this research are to measure the impact of drought stress on WH control with 2,4-D and glyphosate (alone or in combination), the timing of the stress in relation to the herbicide application, and the uptake and translocation of each herbicide. The research hypotheses are: (1) control of drought-stressed WH will be greater with 2,4-D applied alone relative to glyphosate; (2) the timing of the drought stress will alter WH control with both herbicides and tank mixtures; (3) glyphosate uptake and translocation will be lower in drought-stressed WH, but 2,4-D uptake will not be altered.

**Materials and Methods**

*Greenhouse Assays*

Waterhemp (*Amaranthus tuberculatus*) seeds from a population that is neither glyphosate nor 2,4-D resistant (MCR; Ma et al. 2013) were collected and suspended in 0.1 g $L^{-1}$ agar-water solution at 4˚C for at least 30 d to enhance germination. Seeds were germinated in 12-x 12-cm trays with a commercial potting mix (LC1, Sun Gro Horticulture) in the greenhouse. Greenhouse conditions were maintained at 28˚C/22˚C day/night with a 16/8-h photoperiod. Natural sunlight supplemented with mercury halide lamps provided a minimum photosynthetically active radiation (PAR) of 500 µmol $m^{-2}s^{-1}$ photon flux at plant canopy level. Emerged seedlings (2 cm tall) were transplanted into 80-$cm^3$ pots in

the greenhouse. When the seedlings were 4 cm tall, they were transplanted into 950-$cm^3$ pots containing 1:1:1 mixture of potting soil:sand:peat. Slow-release fertilizer (Nutricote, Agrivert, Oxfordshire, UK OX7 4EB) was added to the soil mixture. At this point, WH plants were subjected to drought stress for 7 d until the height of 10 to 12 cm was reached with unstressed plants. Drought stress, indicated by wilting, smaller plants, and less biomass, was created by watering WH plants with a certain amount of water per day. Water was applied to the soil surface as 10, 20, or 40 mL using a graduated syringe at the base of the plant in the first experiment, but only 10 and 40 mL were used in the second experiment to reduce the number of plant samples and the stress level between 10 and 20 mL per day was similar.

Preliminary studies determined that these three watering amounts provided a range of soil moisture levels and corresponding stress levels, as determined by monitoring visual symptoms such as wilting and leaf area, biomass production, height, and soil moisture levels (data not shown). During the first experiment, restricted watering levels occurred throughout the entire study (7 d before and after herbicide application). The second experiment observing the effect of drought timing in relation to herbicide application, placed restricted watering 7 d before or after the application or both 7 d before and after the herbicide application (Table 4.1). Plants that were not restricted to daily water amounts were watered to soil saturation to prevent any moisture stress. Soil moisture content (% volumetric water content) was measured with a soil moisture probe (Hydrosense Model CS260, Campbell Scientific Australia), to a depth of 2.5 cm and 5 cm from each side of the pot, the day of the herbicide application and at harvesting for comparisons among drought scenarios (Table 4.2).

Herbicide treatments (Table 4.3) were applied to WH plants 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. At herbicide

application, plants watered to saturation were 10 to 12 cm tall, 40 mL per day were 8 to 10 cm, and 10 or 20 mL per day were 6 to 8 cm tall. Herbicides were applied at reduced rates to determine the effect of drought stress on weed control without completely controlling the weed with either herbicide alone, as performed in previous studies (Adkins et al. 1998a, 1998b; Tanpipat et al. 1997). Preliminary studies determined that WH injury is induced with a quarter (0.25x or "low") of the field use rate (1120 g active ingredient $ha^{-1}$) with both 2,4-D and glyphosate but does not control the weed. A higher rate of glyphosate, three-quarters of the field use rate (0.75x or "high"), was also used in case 0.25x was not able to induce herbicidal activity because reduced control of drought-stressed plants with glyphosate has been reported. Plants were returned to the greenhouse and were subjected to drought conditions for an additional 7 d. Above ground biomass was harvested and then dried in an oven at 60˚C to determine dry matter.

*Whole-Plant Assay*

After growing WH seedlings and transplanting into 950-$cm^3$ pots in the same manner as the greenhouse assays, WH were watered with 20 mL (water restricted) or to saturation (not restricted) daily. Waterhemp plants were grown under these conditions for 6 days to the height of 6 cm. Plants were then transferred to a growth chamber (16 hour day/8 hour night, 28˚C/26˚C, and PAR 490 $\mu molm^2s^{-1}$) one day prior to applying herbicide treatments. Twenty µM [Uniformly Ring-Labeled (URL)-$^{14}$C]-2,4-D acid (specific activity 1.4 GBq/mmol) or [URL-$^{14}$C]-glyphosate (specific activity 1.9 GBq/mmol) radiolabeled treatments were applied using a glass syringe (Hamilton Co., Reno, NV 89502) to deliver 10 µL (100 Bq) divided into two droplets to the treated leaf. Tween 20 (0.1% v/v, Sigma-Aldrich, St. Louis, MO 63103) was added to both treatments as a surfactant. The fourth leaf (from soil level) was used as the treated leaf and was noted with a marker for clear recognition during harvesting.

After the radiolabeled solution was applied, the plants were returned to the growth chamber until sample collection was conducted at 30 and 48 hours after application (HAA). At sampling, the plant was divided into two parts: the treated leaf and the rest of the plant. The treated leaf was the entire leaf to which the radiolabeled herbicide solution was applied. The treated leaf was immediately rinsed in 3 mL of a water:methanol solution (80:20, v/v) for 45 s to remove any remaining $^{14}C$-herbicide material from the leaf surface in a scintillation vial. The methanol rinse solution was then analyzed via liquid scintillation spectrometry (LSC, Model 1900 TR, Packard Instrument, Meriden, CT 06450) to determine the amount of $^{14}C$-herbicide remaining on the surface. The treated leaf and remaining plant portions were allowed to air dry before they were combusted for 4 min in a biological oxidizer (Model OX-500, R.J. Harvey Instrument Corp., Hillsdale, NJ 07462) and analyzed via LSC to show amount of $^{14}C$-labeled material within each portion. Total radioactive material recovery was determined by summing the amount of $^{14}C$-material recovered from each step and averaged 90% for all experiments.

*Statistical Methods*

Treatments were arranged in a complete randomized design. All experiments were replicated twice, and experiment by treatment interactions were not detected so results were pooled over both experimental replications for all data. The greenhouse assay had five biological replications, and the whole-plant assay had three biological replications for each treatment combination. Dry weights were analyzed as percent of the untreated control for each drought situation. Reductions in WH dry matter were used to indicate greater herbicide activity and efficacy. Data were analyzed using Proc Mixed in SAS (SAS version 9.4, SAS Institute Inc., Cary, NC 27513) using Proc Mixed. Means were separated using Fisher's Protected Least Significant Difference test at $\alpha$=0.05. Herbicide uptake and translocation data were analyzed using SAS in a split-plot design with the whole-plot being each time point and the sub-

plot being the treatment combinations. Means were separated using Fisher's Protected Least Significant Difference test at α=0.05.

**Results and Discussion**

*Waterhemp Control under Drought Conditions*

Waterhemp control varied among the treatments in the first experiment (P=<0.0001, Table 4.4). Glyphosate-low had the greatest amount of dry matter (87% of the untreated control), and all other treatments had similar dry matter levels (Table 4.4). 2,4-D-low had less dry matter than glyphosate-low and equivalent to glyphosate-high, but tank-mixing the two herbicides did not reduce WH dry matter (Table 4.4). The daily water amount had a significant effect on WH dry matter (P=<0.0001, Table 4.5). The greatest amount of dry matter occurred with the lowest water amount (10 mL) and dry matter decreased (*i.e.*, herbicide efficacy increased) with increasing water amounts, except in the saturated water amount (Full) compared to 40 mL per day (Table 4.5).

Reduction in WH dry matter in response to the herbicide treatments followed a similar pattern in the second experiment as measured in the first. A significant difference was detected among the treatments (P=0.0129). Glyphosate-low had the greatest dry matter (95% of untreated) compared to 2,4-D-low and glyphosate-high, which had equivalent amounts (67% and 60% of untreated). The timing of the drought stress in relation to the herbicide application significantly affected waterhemp dry matter production (P=0.0027, Table 4.6). The largest amount of dry matter accumulated (least herbicide efficacy) when the drought stress lasted throughout the entire study (Full) or after the herbicide application (After) (Table 4.6). Waterhemp dry matter was lowest when the drought stress occurred before the application (Before) (Table 4.6). There was a significant herbicide treatment by drought timing interaction (P=<0.0001, Table 4.7). Both 2,4-D-low and glyphosate-low had equivalent WH dry

matter levels when the drought stress occurred either After or Full, but greater dry matter reductions occurred with the Before treatment, within each individual herbicide treatment (Table 4.7). Glyphosate-high had the least amount of WH dry matter with the Before treatment, which was significantly different from the After and Full treatment (Table 4.7). The After treatment had lower WH dry matter accumulation compared to the Full treatment, though (Table 4.7).

Drought stress reduced WH dry matter accumulation when treated with glyphosate (Table 4.4), matching results of previous studies measuring glyphosate efficacy with both grass and dicot weed species (Adkins et al. 1998a, 1998b; de Ruiter and Meinen 1998; Dickson et al. 1990; Kudsk and Kristensen 1992; Tanpipat et al. 1997; Waldecker and Wyse 1985; Wicks and Hanson 1995; Zhou et al. 2007). Drought stress did not alter efficacy of 2,4-D as significantly in comparison with glyphosate, and 2,4-D-low maintained similar WH dry matter levels as glyphosate-high (Table 4.4). Inconsistent results in maintaining weed control levels were also noted with other auxin herbicides in previous studies (Lauridson et al. 1983; Morrison et al. 1995). Increasing drought stress by restricting water provided to WH plants resulted in lower herbicide efficacy (Table 4.5). Drought stress greatly reduced herbicide activity when the drought stress occurred immediately after the herbicide application and for the entire study (Table 4.6). Reduction in dry matter was greater (greater herbicide efficacy) when the drought stress occurred before the herbicide application and plants were watered optimally afterwards (Table 4.6). Therefore, the critical period for reducing herbicide efficacy in WH occurs after the herbicide application but is less important when the stress occurs before the application.

*Uptake and Translocation in Waterhemp under Drought Conditions*

***Uptake*.** There was a significant difference in herbicide uptake among the treatments and presence of drought stress. The amount of 2,4-D uptake (96%) was greater than glyphosate (61%) overall

(P=<0.0001). Waterhemp plants under drought stress had lower herbicide uptake (69%) compared to plants that were not stressed (88%, P=<0.0001). A significant herbicide treatment by drought stress interaction occurred (P=<0.0001). The level of 2,4-D uptake was not different between stressed and unstressed plants, but glyphosate uptake was influenced by drought stress (Table 4.8). Glyphosate uptake reduced from 83% in unstressed plants to 40% in drought-stressed WH plants (Table 4.8).

***Translocation.*** Drought stress had a significant impact on herbicide translocation. More $^{14}$C-material remained in the treated leaf of WH plants treated with 2,4-D (60%) compared to glyphosate (41%) overall (P=<0.0001), but 2,4-D translocation was unaffected by drought stress (Table 4.9). The amount of $^{14}$C-material remaining in the 2,4-D treated leaves of WH plants was equal in stressed and unstressed plants, but stressed WH plants treated with glyphosate had more $^{14}$C-material remaining in the treated leaf compared to unstressed (Table 4.9). With glyphosate, 48% of $^{14}$C-material was located in the treated leaf of drought-stressed WH compared to 36% in the unstressed (Table 4.9).

Drought negatively influenced both uptake and translocation of glyphosate, but did not affect 2,4-D uptake and translocation (Tables 4.8 and 4.9). A reduction in glyphosate uptake and translocation matches previous studies (Ahmadi et al 1980; de Ruiter and Meinen 1998; Klevorn and Wyse 1984; Lauridson et al. 1983; McWhorter et al 1980; Waldecker and Wyse 1985). Maintaining similar uptake levels with 2,4-D in drought-stressed plants has been documented in previous studies (Basler et al. 1961; Hauser 1955; Merkle and Davis 1967; Pallas and Williams 1962), but a difference in 2,4-D translocation between stressed and unstressed plants was not found, which is not consistent with previous work (Basler et al. 1961; Hauser 1955; Merkle and Davis 1967; Pallas and Williams 1962). Lower glyphosate uptake and translocation in drought-stressed WH can contribute to reduced efficacy under field conditions. Plants under drought stress have a different cuticle thickness or wax chemistry compared to unstressed plants (Hatterman-Valenti et al. 2011), which may limit glyphosate uptake by impeding

movement from the outside of the leaf surface inward. Drought may reduce glyphosate translocation due to reduced photosynthesis and photoassimilate transport because less material is being loaded and transported in the phloem of drought-stressed plants (de Ruiter and Meinen 1998). The rate and/or amount of 2,4-D and glyphosate phloem-loading may differ resulting in the measured translocation difference between the herbicides in drought-stressed WH.

With the rates examined in this study, 2,4-D effectively reduced dry matter accumulation of WH under drought stress compared to glyphosate. Greater herbicide efficacy levels occurred when ample water was available after the application, even when plants were drought-stressed prior to the herbicide application. Timing herbicide applications in relation to rainfall events can aid in improving POST herbicide efficacy levels. Uptake and translocation levels of 2,4-D were maintained in stressed plants compared to glyphosate, which demonstrated reduced herbicide uptake and translocation.

Determining how and why 2,4-D maintains consistent uptake and translocation levels in drought-stressed weeds can improve how stressed weeds are controlled, and help determine which POST herbicides should be used during those conditions. Investigating the effect of various adjuvants, formulations (salts and esters), and tank-mix combinations on 2,4-D uptake and translocation in drought-stressed weeds may provide additional information about improving POST weed control during drought conditions. With glyphosate or 2,4-D-resistant weed populations that possess reduced translocation and/or uptake as the mechanism of resistance (Powles and Preston 2006: Riar et al. 2011; Vila-Aiub et al. 2012), determining how drought stress influences glyphosate and/or 2,4-D uptake or translocation individually or in tank-mixes in these resistant populations may be beneficial as well. Even though 2,4-D uptake and translocation were not affected by drought stress in this study, a resistant weed population with reduced 2,4-D uptake and/or translocation may not respond similarly as a sensitive population. Tank-mixing 2,4-D with glyphosate may improve the control of glyphosate-

resistant populations during drought conditions because 2,4-D uptake and translocation are not reduced. Further research into understanding how abiotic stresses influence herbicide efficacy will allow growers to make informed decisions when determining how to manage weeds under various stressful environmental conditions.

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**Tables**

Table 4.1. Description of daily restricted watering scheme used in second greenhouse assay measuring the effect of timing of drought stress on herbicide efficacy.

| Drought Timing | Day 1-7[a] | Day 8-14 |
|---|---|---|
| After | Water to Saturation | 10, 20, or 40 mL |
| Before | 10, 20, or 40 mL | Water to Saturation |
| Full[b] | 10, 20, or 40 mL | 10, 20, or 40 mL |

[a] Herbicide application occurred on day 7.

[b] Full drought timing was used in the first greenhouse assay.

Table 4.2. Soil moisture content of different drought stress timings and water amounts for greenhouse assays.

| | | Soil Moisture Content | |
|---|---|---|---|
| | Water Amount | Herbicide Application[a] | Harvest[a] |
| Drought Timing | mL/Day | % v/v | |
| After | 10 | 32 | 7 |
| | 20 | 28 | 13 |
| | 40 | 30 | 26 |
| Before | 10 | 2 | 36 |
| | 20 | 11 | 30 |
| | 40 | 25 | 29 |
| Full | 10 | 5 | 5 |
| | 20 | 10 | 11 |
| | 40 | 23 | 18 |

[a] Soil moisture content of untreated plants watered to saturation at herbicide application was 34% and 30% at harvest.

Table 4.3. Herbicide treatment list for greenhouse assays to measure waterhemp dry matter accumulation under drought stress.

| Experiment 1 | Experiment 2 |
|---|---|
| 2,4-D (0.25x)[a] | 2,4-D (0.25x) |
| Glyphosate (0.25x)[b] | Glyphosate (0.25x) |
| 2,4-D (0.25x) + Glyphosate (0.25x) | Glyphosate (0.75x) |
| Glyphosate (0.75x)[b] | |
| 2,4-D (0.25x) + Glyphosate (0.75x) | |

[a] 2,4-D (266 g ae/ha, Dow AgroSciences, Indianapolis, IN 46268)

[b] Glyphosate (280 or 840 g ae/ha, Durango 4AE, Indianapolis, IN 46268)

Table 4.4. Dry matter as percent of untreated control of herbicide treatments from first greenhouse assay measuring effect of drought stress on waterhemp control.

| Treatment[b] | Dry Weight<br>% of Untreated Control[a] |
|---|---|
| 2,4-D (0.25x) | 64.7 b |
| Glyphosate (0.25x) | 87.2 a |
| 2,4-D + Glyphosate (0.25x, 0.25x) | 61.4 b |
| Glyphosate (0.75x) | 62.1 b |
| 2,4-D + Glyphosate (0.25x, 0.75x) | 57.2 b |
| P-value | <0.0001 |

[a] Treatment means followed by the same letter are not statistically different by Fisher's Least Significant Difference test at $\alpha=0.05$.

[b] 2,4-D (266 g ae/ha) and glyphosate (280 or 840 g ae/ha).

Table 4.5. Waterhemp dry matter as influenced by daily water amount from the first greenhouse assay, designed to measure the effect of drought stress on herbicide efficacy.

| Water Amount | Dry Weight |
|---|---|
| mL/Day | % of Untreated Control |
| 10 | 97.8 a |
| 20 | 69.6 b |
| 40 | 44.1 d |
| Full | 54.6 c |
| P-value | <0.0001 |

[a] Treatment means followed by same letter are not statistically different by Fisher's Least Significant Difference test at $\alpha$=0.05.

Table 4.6. Effect of drought stress timing on waterhemp dry matter from second greenhouse assay.

| Timing of Drought Stress[a] | Dry Weight<br>% of Untreated Control[b] |
|---|---|
| After | 79.3 a |
| Before | 53.7 b |
| Full | 90.0 a |
| P-value | 0.0027 |

[a] Timing of drought stress occurred 7 d before herbicide application (Before), 7 d after application (After), or both 7 d before and after application (Full). Plants were watered to saturation when not under drought stress.

[b] Treatments followed by same letter are not statistically different by Fisher's Least Significant Difference test at α=0.05.

Table 4.7. Herbicide treatment by drought timing interaction from the second greenhouse assay designed to determine the effect drought stress on herbicide efficacy.

| Treatment | Timing of Drought Stress[a] | Dry Weight % of Untreated Control[b] |
|---|---|---|
| 2,4-D (0.25x)[c] | After | 70.5 c |
| | Before | 47.6 de |
| | Full | 83.1 bc |
| Glyphosate (0.75x)[c] | After | 54.9 d |
| | Before | 40.3 e |
| | Full | 85.0 bc |
| Glyphosate (0.25x)[c] | After | 112.6 a |
| | Before | 73.2 c |
| | Full | 102.0 ab |
| | P-value | <0.0001 |

[a] Timing of drought stress occurred 7 d before herbicide application (Before), 7 d after application (After), or both 7 d before and after application (Full). Plants were watered to saturation when not under drought stress.

[b] Treatments followed by same letter are not statistically different by Fisher's Least Significant Difference test at α=0.05.

[c] Herbicide treatments (0.25x) and (0.75x) are referred as low and high in text

Table 4.8. Glyphosate and 2,4-D uptake levels as influenced by drought stress from whole-plant assay with waterhemp.

| Herbicide | Drought Stress[a] | Herbicide Uptake |
|---|---|---|
| | | % of Recovered $^{14}C$-Material[b] |
| 2,4-D | Yes | 98.0 a |
| | No | 94.6 a |
| Glyphosate | Yes | 40.3 c |
| | No | 83.0 b |

[a] Waterhemp plants were watered with 20 mL per day for drought stress (Yes) or to saturation for no stress (No).

[b] Treatments followed by same letter are not statistically different by Fisher's Least Significant Difference Test α=0.05.

Table 4.9. The amount of $^{14}C$-material remaining in the treated leaves of waterhemp plants growing with or without drought stress from the whole-plant assay.

| Herbicide | Drought Stress[a] | $^{14}C$-Material in Treated Leaf: % of $^{14}C$-Material Absorbed[b] |
|---|---|---|
| 2,4-D | Yes | 56.9 ab |
| | No | 64.9 a |
| Glyphosate | Yes | 47.9 b |
| | No | 35.7 c |

[a] Waterhemp plants were watered with 20 mL per day for drought stress (Yes) or to saturation for no stress (No).

[b] Treatments followed by same letter are not statistically different by Fisher's Least Significant Difference Test $\alpha$=0.05.

# CHAPTER 5

## SYNOPSIS OF RESEARCH FINDINGS AND IMPACTS

The synthetic auxin herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), is one of the oldest and most widely used herbicides globally. Since its development in the 1940s, a tremendous amount of research has been conducted on 2,4-D, but the majority of the research was conducted prior to the 1970s. The use of 2,4-D is expected to increase in the near future due to the development of new resistant crop varieties (Egan et al. 2011; Mortensen et al. 2012). The need for research utilizing contemporary methods and tools is necessary with the increase in usage.

This research utilized two different growth chamber assays to measure uptake, translocation, and metabolism of herbicides as affected by several different factors. The whole-plant assay provided information on 2,4-D and glyphosate uptake and translocation. Uptake and translocation of 2,4-D was measured in Enlist and non-transformed (NT) corn and soybean isogenic lines (varieties) to determine if the injury observed in Enlist soybean is related to changes in 2,4-D uptake and translocation, and investigate how the components of Enlist Duo influence these two factors. The whole-plant assay determined 2,4-D uptake when using a tank-mix of Enlist Duo and acetochlor after injury was witnessed in Enlist corn. Uptake and translocation of 2,4-D and glyphosate were measured in waterhemp plants under drought stress to determine any differences between the two herbicides. The excised-leaf assay determined the metabolism of 2,4-D in Enlist and NT soybean and corn varieties, and the metabolic fate of 2,4-D, dichlorophenol (DCP), and 2,4-D ester. Metabolism of 2,4-D was studied in Enlist crops to determine if there was any difference between Enlist and NT varieties and if glyphosate alters 2,4-D metabolism. The metabolism of DCP was determined as a comparison to the metabolic fate of DCP and 2,4-D in Enlist and NT crops. Metabolism of 2,4-D ester was measured in Enlist soybean to determine

why injury does not develop when Enlist soybean are treated with 2,4-D ester compared to 2,4-D choline. These two simple assays measure the uptake, translocation, and metabolism of herbicides and can be adapted to determine these factors in relation to many other variables and including herbicide resistance and environmental stresses.

The injury observed in Enlist soybean was attributed to excessive 2,4-D foliar concentrations immediately after an application of Enlist Duo due to increased uptake. Injury to Enlist corn is less frequent because the uptake of 2,4-D is significantly lower, especially with the Enlist Duo formulation, as well as endogenous detoxification mechanisms unlinked to the transgene. Increasing the uptake of 2,4-D in Enlist corn by tank-mixing Enlist Duo with formulated acetochlor resulted in injury similar to Enlist soybean. Conversely, the proherbicide 2,4-D ester formulation eliminated injury to Enlist soybean by reducing the cellular concentration of 2,4-D acid. In Enlist soybean and corn, 2,4-D is metabolized in the same fashion as DCP because 2,4-D is converted to DCP by the aryloxyalkanoate dioxygenase (AAD) enzymes in Enlist crops, but this is not the case in NT varieties. In Enlist soybean, LC-MS/MS identified two metabolites. This research measured the formation of large DCP-sugar conjugates, and both metabolites displayed intermediate metabolites identified during the metabolism of DCP in other plant species, indicating further DCP metabolism than had been previously determined.

It was concluded that injury to Enlist crops is linked to excessive 2,4-D concentrations in the plant cells due to increased foliar 2,4-D uptake. Injury to Enlist soybean is more common because greater 2,4-D uptake occurs rapidly and only one 2,4-D detoxification pathway is present compared to Enlist corn, and injury can be eliminated by temporarily reducing the cellular concentration of 2,4-D acid with the 2,4-D ester formulation. Reducing crop uptake levels or using a different formulation of 2,4-D can influence the risk of crop injury, however, these mechanisms may come with drawbacks. Reducing uptake in the crop may also reduce uptake in weeds as well as lower efficacy, and using the ester

formulation of 2,4-D has many negative aspects in comparison to the choline formulation, such as off-target movement to sensitive plants (Grover et al. 1972; Kelley et al. 2005). Balancing crop uptake, weed control, and crop injury will be required with the use of metabolism-based herbicide resistance traits now and in the future.

The impact of drought stress on 2,4-D and glyphosate efficacy was studied in different greenhouse experiments, which determined that 2,4-D has greater efficacy on waterhemp than glyphosate under drought conditions at reduced rates. The timing of the stress in relation to the herbicide application was also investigated. Both herbicides achieved greater herbicide efficacy when plants were watered to saturation after the herbicide application, even if the waterhemp plants were subjected to drought stress prior to the application. However, when waterhemp plants were subjected to drought stress immediately after the herbicide application, control levels with both herbicides were equivalent to when the drought occurred both before and after the application. The uptake and translocation of both herbicides were measured to better understand why waterhemp control under drought stress was greater with 2,4-D. Uptake and translocation of 2,4-D were equivalent between stressed and unstressed plants, while drought stress reduced glyphosate uptake and translocation. The greater reduction in dry matter accumulation of drought-stressed waterhemp with 2,4-D relative to glyphosate at reduced rates is attributed to maintaining uptake and translocation of the herbicide in stressed plants. Including 2,4-D in tank-mixtures can aid growers when controlling drought-stressed weeds, and whenever possible, appropriate timing of postemergence (POST) herbicide applications in relation to rainfall events can also improve herbicide efficacy. Making a POST application prior to rain can increase weed control, even with stressed plants.

There were limitations to this research. The injury to Enlist crops was attributed to excessive 2,4-D uptake levels, but the difference in uptake between soybean and corn with Enlist Duo was not

explained. The difference between soybean and corn may be related to the anatomical differences between dicots and monocots, which might also be measured when comparing 2,4-D uptake in other dicot/monocot species. Alternatively, a specific feature or characteristic of soybean or corn leaf morphology, physiology, or cuticle chemistry may influence uptake. This research did not include a weed control portion, which could influence whether or not 2,4-D uptake levels can be altered in the crop. For example, if 2,4-D uptake in dicot weeds is equal to what was determined in soybean, uptake levels could be reduced and still maintain equivalent weed control levels and lower the risk of crop injury.

This research did not investigate other 2,4-D salt formulations (besides choline). Choline is a metabolite in plants required in the synthesis of membrane phospholipids and for the metabolite, glycine betaine, which is important in conferring tolerance to several abiotic stresses including drought, salinity, UV-radiation, and heat (Kreslavski et al. 2001; McNeil et al. 2001). Exogenous applications of choline to plants may induce physiological responses, including stress-related processes and synthesis of phospholipids, which could contribute to the observed injury to Enlist crops. Further research on the use of alternative 2,4-D salt formulations and the development of injury in Enlist crops will determine if the injury is independent of the choline salt.

This research did not determine how the necrotic injury to Enlist soybean forms or measure the levels of 2,4-D at the cellular level. The necrotic spots could relate to the hypersensitive response and/or excessive ethylene and/or ABA levels, or localized accumulation of reactive oxygen species, but this research did not investigate these possibilities. Also, the chemical nature of 2,4-D in the necrotic lesions was not determined. The forms of 2,4-D present in the lesions (either as metabolites or the parent free acid or both), and determining which form(s) are more prevalent during a time course, could help to explain how, why, and where the injury develops. Uptake of 2,4-D was measured at a whole-

plant level but not at an intracellular concentration level. Determining the amount of 2,4-D present in the cell after an herbicide application may have greater meaning than the amount measured in intact plant tissues or organs in this research. Since a large percentage of the 2,4-D in Enlist crops will be rapidly metabolized, quantifying the amount of remaining free acid is more important toward explaining the injury than organ or tissue uptake levels measured in this research.

Greater herbicide efficacy in waterhemp under drought stress occurred when ample water was supplied after the herbicide application, even with plants that experienced severe stress prior to the application. Increased herbicide uptake and/or translocation or another factor could be why herbicide efficacy was greater under these conditions but this research did not investigate these factors. The uptake and translocation of 2,4-D was similar between drought-stressed and unstressed plants, but less uptake and translocation of glyphosate was detected. Differences in uptake and translocation between the two herbicides were not explained by this research, but could be due to dissimilarities in the physiochemical factors inherent to each herbicide or environmental-chemical interactions between the drought-stressed plant and each herbicide. Future research investigating the various physiochemical factors of each herbicide (pKa, lipophilicity, ionic state, etc.) and the cuticle thickness and wax composition of drought-stressed plants will help to answer questions created by completing the current research.

With the potential for an increase in the use of 2,4-D and other auxin herbicides, more research will be needed on 2,4-D pertaining to transgenic crops, weed control, tank-mixtures, adjuvants, resistant weed populations, and interactions with the environment (Mortensen et al. 2012). More detailed research can be conducted with 2,4-D than what is currently being conducted by weed scientists and plant biologists as the knowledge and understanding of how natural auxins and 2,4-D function and interact within the plant increases. Such basic research investigations may include identifying and

characterizing auxin receptors in dicots other than *Arabidopsis*; revealing which auxin receptor(s) bind synthetic auxin herbicides and how these interactions lead to lethality in dicot weeds; determining how the natural state/concentration of various plant hormones under varying environmental conditions may interact (synergistic, antagonistic, or no influence) with the hormonal response created by auxin herbicides. The near future may see an increase in physiological research with 2,4-D and other synthetic auxin herbicides, similar to the amount of research conducted on 2,4-D when it was initially released in the 1940s and commonly used in the 1950s and 1960s.

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