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Stewarding 2,4-D- and dicamba- based weed control technologies in cotton and soybean production systems

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By

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Distinguishing 2,4-D and dicamba herbicide formulations in cotton and soybean tissue is challenging in regulation of crop injury from these herbicides. Additionally, stewardship of 2,4-D and dicamba technologies is important to maximize their longevity and efficacy. Research was conducted to (1) characterize cotton and soybean response to various formulations of 2,4-D or dicamba with or without glyphosate, (2) develop a method for classifying these formulations in crop tissue, and (3) optimize use of chloroacetamide herbicides in dicamba systems for mitigation of selection pressure on dicamba. Formulations evaluated include dicamba diglycolamine (DGA), dimethylamine (DMA), N,N-Bis-(3-aminopropyl) methylamine (BAPMA), and DGA plus potassium acetate (KAc); and 2,4-D DMA, acid, isooctyl ester (ESTER), and choline. Weed management by the chloroacetamides *s*-metolachlor and acetochlor was evaluated with applications preemergence (PRE), early postemergence (EP), late postemergence (LP), PRE followed by (fb) EP, PRE fb LP, and EP fb LP.

Cotton and soybean response differed by 2,4-D and dicamba formulation, and glyphosate presence. Cotton yield was reduced by 200 to 500 kg ha^{-1} following exposure to 2,4-D choline or DMA relative to acid or ESTER. Glyphosate presence led to a reduction in cotton and soybean

yield of 377 and 572 kg ha^{-1}, respectively. Exposure to dicamba DMA resulted in a 263 kg ha^{-1} reduction in soybean yield relative to dicamba DGA, and glyphosate presence reduced yield by 439 and 246 kg ha^{-1} in cotton and soybeans, respectively. Chemometric analyses generated models capable of up to 85% accuracy in identifying dicamba formulation in cotton and soybean tissue, and up to 80% accuracy in identifying 2,4-D formulation.

Split chloroacetamide applications improved cotton yield up to 60%, reduced weed densities up to 90%, and improved control up to 56% relative to single applications. Cotton height was reduced up to 23% if a single chloroacetamide application was made. Soybean yield was maximized following any chloroacetamide application timing except PRE alone, and weed control was reduced up to 31% following single chloroacetamide application relative to split applications. These results will aid regulatory bodies in managing use of new weed control technologies and will assist producers in stewarding these new technologies.

DEDICATION

I dedicate this work to the two people who have walked with and often carried me through one of the most challenging periods of my life:

"I believe in the sand beneath my toes/The beach gives a feeling, an earthy feeling
I believe in the faith that grows/And the four right chords can make me cry
When I'm with you I feel like I could die and that would be alright, alright"

For Annah, my abejorra

"Let me tell you a secret about a father's love/A secret that my daddy said was just between us
He said daddies don't just love their children every now and then
It's a love without end, amen/It's a love without end, amen"

For Gamma Γ

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CHAPTER I

DISSERTATION BACKGROUND

Introduction

There are currently 255 reported herbicide-resistant (HR) weed species globally (Heap 2019), and continued development of new species with resistance remains one of the greatest challenges to crop production. Glyphosate-resistant (GR) weeds are a large component of the HR weed problem, as there are currently 43 reported GR weed species, and more populations are documented each year (Heap 2019). The spread of weed species such as Palmer amaranth (*Amaranthus palmeri* S.Wats), waterhemp (*Amaranthus tuberculatus* [Moq.] Sauer), and kochia (*Bassia scoparia* [L.] A.J. Scott) with multiple-resistance to several herbicide modes-of-action (MOA) such as glyphosate, protoporphyrinogen IX oxidase inhibitors, and acetolactate synthase inhibitors has made weed management an increasingly difficult component of crop production systems (Bagavathiannan and Norsworthy 2013; Crespo et al. 2014; Culpepper et al. 2010; Eberlein and Fore 1984; Forcella 1985; Stubbendieck et al. 2003; Webster 2012, 2013). New weed control biotechnologies have been developed and commercialized to address herbicide resistance. These new biotechnologies are based on the use of cotton (*Gossypium hirsutum* L.), soybean (*Glycine max* L. [Merr.]), and corn (*Zea mays* L.) cultivars that contain engineered resistance to the auxin herbicides 2,4-D or dicamba. In the case of the Roundup Ready® XTEND Crop System (Bayer CropSciences, Whippany, NJ 07981), cotton and soybean cultivars are available with engineered resistance to the auxin herbicide dicamba, conferred via a transgene

encoding dicamba monooxygenase originally taken from the soil bacteria *Pseudomonas maltophilia* (Behrens et al. 2007; Feng and Brinker 2014). The cotton cultivars in the XTEND™ system also express resistance to glyphosate and glufosinate, and the soybean cultivars express resistance to glyphosate. Similarly, the Enlist™ Weed Control System (Dow AgroSciences, Indianapolis, IN 46268) features cotton, corn, and soybean cultivars with engineered resistance to 2,4-D, glyphosate, and glufosinate (Richburg et al. 2012; Wright et al. 2010). While cultivars with tolerance to glyphosate or glufosinate have been available for some time, 2,4-D resistance in these crops is novel and conferred by an aryloxyalkanoate dioxygenase that metabolizes 2,4-D in vivo (Richburg et al. 2012). These new weed control technologies have been widely adopted, with up to 60% of the area used for cotton and soybean production in some regions being planted to dicamba-resistant cultivars (Lingenfelter et al. 2017; USDA 2017). Producers have widely adopted these technologies in order to access elite germplasm, protect their crops against off-target movement (OTM) of auxin herbicides such as dicamba and 2,4-D, and in order to utilize 2,4-D and dicamba for postemergence (POST) control of difficult-to-manage weeds (Egan et al. 2014; Mortensen et al. 2012). The ability to utilize 2,4-D and dicamba POST is a useful tool for weed management given their ability to efficaciously control a broad spectrum of dicotyledonous weed species and the relatively low incidence of weeds resistant to auxin herbicides (Heap 2019). However, widespread adoption of these technologies has not come without challenges.

One concern with increased use of 2,4-D and dicamba is that due to the highly-efficacious nature of these herbicides and their MOA as synthetic plant hormones, they are highly injurious to susceptible species such as off-target susceptible crops, ornamentals, trees, and other plants, even at very dilute sub-lethal concentrations. Furthermore, these herbicides are inherently prone to OTM via physical herbicide spray drift, spray equipment contamination,

herbicide volatilization, and temperature inversions (Boerboom 2004; Cundiff et al. 2017; Egan et al. 2014; Mortensen et al. 2012). As such, the increased use of these herbicides has already led to a multitude of auxin herbicide-injury on susceptible crops and off-target areas across millions of acres in 2017 and 2018 (Bradley 2017, 2018). The trouble with managing reports of OTM leading to crop injury is that while only a select few herbicides are labeled for use in these new technologies, several more are available for other uses such as weed control in range and pasture or rights-of-way. This leads to the possibility of illegal use of a non-labeled herbicide causing injury to susceptible crops. However, the response in terms of visible symptomology of susceptible soybeans and cotton is nearly identical following exposure to all herbicide products containing 2,4-D or dicamba, regardless of whether they are labeled or not (Johnson et al. 2012; Marple et al. 2007; Sciumbato et al. 2004). Additionally, 2,4-D and dicamba are often used in a tank-mix with glyphosate, which may affect crop response. In order to better manage cases of crop injury following OTM of 2,4-D or dicamba, a better understanding of crop response to different formulations of these products is required. Furthermore, the use of analytic techniques such as infrared spectroscopy and chemometrics may enable creation of classification models capable of identifying discrete auxin herbicide formulations present in damaged cotton and soybean tissue. A joint approach utilizing chemometric analyses such as principal component analysis and linear discriminant analysis with infrared spectroscopy has been used successfully in past applications such as classifying Thai wines and detecting weeds in cabbage (*Brassica oleracea* L.) fields (Deng et al. 2016; Lee et al. 2009).

An additional concern with increased 2,4-D and dicamba use is properly stewarding these technologies in an effort to prolong their weed control efficacy by prolonging the onset of herbicide resistance to auxin herbicides. One commonly-recommended method for prolonging

the onset of resistance is to mitigate selection pressure on a given herbicide MOA. This can be achieved by the use of herbicides with multiple MOA tank-mixed and in rotation (Beckie and Reboud 2009; Gressel and Segel 1990; Powles et al. 1997; Wrubel and Gressel 1994). Tank-mixing two herbicides can add up to four years before the onset of herbicide resistance development, as compared to rotating herbicides each year (Powles et al. 1997). If 2,4-D and dicamba are used repeatedly and without any rotation or diversification of weed control methods, Neve et al. (2011) demonstrated that 4,000 Palmer amaranth plants producing 250,000 seeds plant^{-1} would be capable of producing five seeds with herbicide resistance if the mutation rate for resistance to a given herbicide is at least five per one billion individuals. Palmer amaranth and similar weeds are capable of reproducing at and beyond such magnitude (Culpepper et al. 2010; Webster 2012, 2013). Furthermore, kochia populations with limited or no sensitivity to dicamba have already been reported in Nebraska and the Midwestern U.S. (Cranston et al. 2001; Crespo et al. 2014; Preston et al. 2009). While the current incidences of 2,4-D- and dicamba-resistant weed species are relatively low (Heap 2019), producers must utilize a proactive, diversified approach to chemical management of weeds in order to steward new weed control technologies (Meyer et al. 2015). Chloroacetamide herbicides such as *s*-metolachlor and acetochlor are promising candidates for use in dicamba- and 2,4-D- based weed control systems due to their high residual (preemergence, PRE) control of small-seeded dicotyledonous weeds such as Palmer amaranth, waterhemp, and kochia (including GR populations), and the flexibility with which producers can utilize them (labeled for use at a wide range of concentrations and application timings and safe on most crops). Chloroacetamide herbicides are labeled for use in tank-mixes with the new weed control technologies, and function by preventing germination of weed seeds, thus reducing the number of emerged weeds requiring application of a POST

herbicide such as 2,4-D or dicamba, effectively reducing or eliminating selection pressure on the auxin herbicides. However, due to the vast range of concentrations and application timings at which chloroacetamides can be used, a better understanding of how to properly utilize these herbicides in terms of use rate, application timing, and herbicide selection (*s*-metolachlor or acetochlor) is necessary.

While the availability of the new weed control technologies based upon the use of 2,4-D and dicamba adds a much-needed tool to the weed control arsenal, there are still many challenges to work out with their adoption. An additional factor affecting the adoption of these technologies is a resurgent cotton market leading to multiple producers entering cotton production for the first time. Cotton production has historically been a generational endeavor, so many of the lessons learned in the cotton production of the past may need to be revisited with the availability of 2,4-D- and dicamba-based technologies.

With the aforementioned considerations, the objectives of this dissertation research are sevenfold:

(1) To identify differences in cotton and soybean response at the field level to sub-lethal concentrations of various formulations of 2,4-D with or without glyphosate.

(2) To identify differences in cotton and soybean response at the field level to sub-lethal concentrations of various formulations of dicamba with or without glyphosate.

(3) To apply Fourier-Transform infrared spectroscopy and chemometrics towards creating a classification model capable of identifying discrete 2,4-D product

formulations present in cotton and soybean tissue damaged by off-target deposition of a sub-lethal concentration.

(4) To apply Fourier-Transform infrared spectroscopy and chemometrics towards creating a classification model capable of identifying discrete dicamba product formulations present in cotton and soybean tissue damaged by off-target deposition of a sub-lethal concentration.

(5) To optimize chloroacetamide herbicide use in dicamba-resistant cotton production systems for control of glyphosate-resistant Palmer amaranth and herbicide technology stewardship.

(6) To optimize chloroacetamide herbicide use in dicamba-resistant soybean production systems for control of glyphosate-resistant Palmer amaranth, waterhemp, and kochia and herbicide technology stewardship.

(7) Appendix: To provide a brief history of weed control in cotton production systems with a focus on the use of auxin herbicides such as 2,4-D and dicamba.

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CHAPTER II

APPLICATION OF FTIR SPECTROSCOPY AND CHEMOMETRICS FOR THE CLASSIFICATION OF DICAMBA FORMULATIONS IN DAMAGED COTTON AND SOYBEAN TISSUE

Abstract

Increased use of dicamba in row crop production has led to multiple reports of damage to susceptible cotton and soybeans following off-target movement (OTM). Research was conducted in 2017 and 2018 in Starkville, MS to develop a chemometrics and spectroscopy method to create a classification model capable of identifying specific dicamba formulations present in damaged crop tissue. Dicamba diglycolamine (DGA), dimethylamine (DMA), N,N-Bis-(3-Aminopropyl) methylamine (BAPMA), and diglycolamine with potassium acetate (DGAKAC) were applied to susceptible cotton and soybeans at 35, 18, 9, 4, 2, and 1 g dicamba ae ha^{-1}, and samples were analyzed with infrared spectroscopy, which were further analyzed using principal component analysis (PCA) and linear discriminant analysis (LDA). Joint PCA-LDA models were only capable of classifying dicamba formulation with 40% accuracy, whereas LDA alone was 80 to 85% accurate. Models performed worst when classifying dicamba DMA (27% to 80% accuracy), and best when classifying dicamba DGA/DGAKAC (40 to 85% accuracy). Correct classification of dicamba DGA in the presence of dicamba DGAKAC (and vice-versa) was reduced relative to other formulations, likely due to similarity of the molecular structure of DGA and DGAKAC. This research suggests that with further refining, chemometric analysis of

spectral data from damaged crop tissue may be an economical, efficient, and promising application to support management of crop injury cases following OTM of dicamba.

Nomenclature: Cotton, *Gossypium hirsutum* L.; dicamba; diglycolamine; dimethylamine; N,N-Bis-(3-Aminopropyl) methylamine; potassium acetate; soybean, *Glycine max* L. [Merr.]

Key words: chemometrics, cotton, dicamba, formulation, off-target movement, soybeans

Introduction

The development of herbicide-resistant (HR) weeds continues to be one of agriculture's greatest challenges, with 255 HR weed species being reported as of 2019 (Heap 2019). Of these 255 HR weed species, 43 are glyphosate-resistant (GR) and more populations are reported each year (Heap 2019). New weed control technologies have been commercialized with hopes to control these increasingly difficult-to-manage species. Bayer CropScience's (Bayer Corporation, 100 Bayer Boulevard, Whippany, NJ 07981) Roundup Ready® Xtend Crop System includes cotton (*Gossypium hirsutum* L.) cultivars with engineered resistance to glufosinate, glyphosate, and dicamba, and soybean (*Glycine max* L. [Merr.]) cultivars with resistance to glyphosate and dicamba (Behrens et al. 2007; Feng and Brinker 2014). Resistance to dicamba is conferred via a dicamba monooxygenase (DMO) transgene taken from the soil bacteria *Pseudomonas maltophilia* and engineered into cotton and soybean lines (Behrens et al. 2007; Feng and Brinker 2014). Adoption of dicamba-resistant cotton and soybean technology has been widespread, especially in the mid-southern United States where over 60% of the area planted is to cotton with dicamba technology (USDA 2017), and in other regions where large areas are planted to dicamba-resistant soybeans (Lingenfelter et al. 2017). High rates of adoption will likely continue to facilitate access to elite germplasm, to control HR weed species, and to protect against off-target deposition (OTD) of dicamba (Egan et al. 2014; Mortensen et al. 2012). Dicamba use in

this system is promising due to demonstrated efficacy and the relatively low incidence of dicamba-resistant weed species (Heap 2019). Increased dicamba use following commercialization of this technology has led to multiple reports of crop damage resulting from OTD of dicamba onto susceptible crops. By October 15, 2017, there were reports of approximately 1.5 million ha of U.S. soybeans damaged by dicamba (Bradley 2017). Similarly, by July 15, 2018, an estimated 445,000 ha of soybeans in the U.S. were injured by dicamba (Bradley 2018). The sensitivity of soybean and cotton to dicamba is well-documented (Behrens and Lueschen 1979; Egan et al. 2014; Marple et al. 2007; Sciumbato et al. 2004). Generally, cotton and soybeans respond to dicamba exposure with conspicuous visible injury symptoms including leaf cupping and strapping, stem and petiole epinasty, changes in height and node spacing, and callus formation on stems (Buol et al. 2018; Egan et al. 2014; Marple et al. 2007; Sciumbato et al. 2004). Unfortunately, off-target movement (OTM) of dicamba onto susceptible crops can occur in a multitude of ways, including volatility, drift, contamination of spray equipment, and temperature inversion events (Boerboom 2004; Cundiff et al. 2017; Egan et al. 2014; Mortensen et al. 2012). Further complicating the issue is the availability of multiple dicamba-containing products on the herbicide market. Three dicamba-containing products are labeled for use in the Roundup Ready® Xtend Crop System: (1) Xtendimax® herbicide with VaporGrip® Technology and (2) Engenia™ (BASF Corporation, 100 Park Ave., Florham Park, NJ 07932), and (3) FeXapan™ (DuPont USA, 1007 Market Street, Wilmington, DE 19898). However, many other dicamba-containing products are available for use in other production systems including pre-plant burndown weed control, range and pasture weed control, and weed control in rights-of-way. The availability of multiple dicamba-containing herbicides for legal use in other applications opens the possibility of potential illegal use in the Roundup Ready® Xtend

Crop System. Older dicamba formulations not labeled for use in the Roundup Ready® Xtend Crop System such as dicamba dimethylamine (DMA) often demonstrate higher rates of volatility and proclivity for herbicide drift than those labeled for use (Behrens and Lueschen 1979; Gavlick 2015). Previous research has demonstrated slight differences in susceptible crop response to different formulations of the same auxin molecule, depending on crop and herbicide. Thompson et al. (2007) reported 7% greater soybean injury following exposure to 2,4-D ester relative to 2,4-D amine. Sosnoskie et al. (2014) found reduced cotton height and increased injury following exposure to 2,4-D ester as opposed to 2,4-D amine or 2,4-D choline. Bauerle et al. (2015) reported increased tomato (*Solanum lycopersicum* L.) sensitivity to 2,4-D ester relative to 2,4-D dimethylamine or 2,4-D acid. Similarly, Dias et al. (2014) found increased injury to tomato and sunflower (*Helianthus annuus* L.) following exposure to triclopyr trimethylamine relative to triclopyr butoxyethyl ester, triclopyr pyridinyloxyacetic acid, or triclopyr choline; and increased soybean injury following exposure to triclopyr trimethylamine or triclopyr choline relative to the other triclopyr formulations.

However, susceptible crop response is similar to all dicamba herbicides and only differs slightly in magnitude, though these differences are very unlikely to be detectable by observation at the field level (Buol et al. 2019), thus necessitating an alternative method to identify specific dicamba formulations (and thus products) present in damaged crop tissue. Analytic techniques such as high-performance liquid chromatography/mass spectrometry, gas chromatography/mass spectrometry, and inductively coupled plasma optical emission spectrometry have been utilized to detect and identify low concentrations of analytes in various media ranging from dicamba in small plastic reservoirs (Gavlick et al. 2015), phenylureas in plant tissue (Peña et al. 2002), and soybean seeds (Duke et al. 2018). These techniques are not feasible for identifying the

formulation molecules due to sample extraction methods that cleave the salt groups of the formulation molecule (diglycolamine salt, dimethylamine salt, etc.) from the parent dicamba molecule, thus making it impossible to identify (Reid 2017). Thus, while these methods are efficient at detecting and quantifying dilute concentrations of various herbicide molecules such as 2,4-D and dicamba, alone or in mixtures, they are not feasible for identifying formulated salts coupled with said herbicide molecules. An alternative analytic method that is also considerably cheaper and faster than those aforementioned is Fourier-Transform infrared spectroscopy (FTIR). The advantage of FTIR spectroscopy is that it requires little or no sample preparation before analysis, allowing ground plant tissue to be analyzed.

FTIR spectroscopy involves characterizing the interaction between infrared (IR) radiation and a liquid, solid, or gaseous sample (Simonescu 2012). By measuring the pattern and magnitude of infrared light absorbed by a sample, inferences can be made about its contents. The absorbance spectrum produced by FTIR plots relative sample absorbance (%) by frequency in reciprocal centimeters (cm^{-1}, 'wavenumbers'), which have a reciprocal relationship with wavelength. An infrared spectrum can help predict the chemical composition of the sample based on the assumption that specific chemical functional groups absorb radiation at different frequencies (Simonescu 2012). Previous research has demonstrated the ability of FTIR spectroscopy to detect and identify trace amounts of chemical compounds in various media (Simonescu 2012). Puckrin et al. (1996) were able to characterize gas concentrations in the troposphere and stratosphere using FTIR spectrometers; and other research has used modified FTIR spectroscopy to identify pollutants and other chemical compounds in environmental samples (Simonescu 2012). Lee et al. (2009) utilized FTIR spectroscopy with chemometric analyses to successfully classify Thai wine samples based upon chemical content. Similarly,

Deng et al. (2016) utilized FTIR and chemometric analysis to correctly identify weeds in cabbage (*Brassica oleracea* L.) fields with up to 84% accuracy. Infrared spectroscopy is often combined with statistical analyses such as Principal Component Analysis (PCA), Linear Discriminant Analysis (LDA), or joint PCA-LDA to glean information and build classification models from spectral data in a process called chemometrics (Ami et al. 2010; Deng et al. 2016; Lee et al. 2009; Lehmann et al. 2015; Reid 2017; Simonescu 2012). PCA is a modeling procedure that clarifies sample-variable relationships by dimensional reduction, a process that transforms data into a new matrix with a condensed number of variables (dimensions) called Principal Components (PC, also called eigenvectors). PCs are linear combinations of the original variables and are constructed such that they are orthogonal to each other. The goal of generating PCs is to create a new coordinate system that captures the maximum amount of variation in the original dataset. Subsequent PCs are created such that the first PC captures the greatest amount of variation, the second PC captures the second most, and so on until converging at 100% explained variation. Altogether, the total amount of variation captured by all PCs is the total explained variation, or the variation considered by the classification model. Similarly, LDA is a supervised classification method used to identify unknown samples based on a given set of known samples used to 'train' the classification model. Joint PCA-LDA creates a model that utilizes the reduced number of dimensions created in PCA to consider the within and between group variances in a dataset, and ultimately use these parameters to predict unknown sample classification (Ami et al. 2010; Reid 2017). Reid (2017) constructed preliminary PCA-LDA classification models with up to 98% accuracy in classifying dicamba formulations (diglycolamine salt, dimethylamine salt, N,N-Bis-(3-Aminopropyl) methylamine salt, and diglycolamine salt with potassium acetate) in damaged soybean tissue harvested 0, 1, 3, 7, 21,

and 28 DAT that had been treated with 17.5 g dicamba ae ha^{-1}. However, these models only considered one dicamba concentration (relative to the wide range of potential concentrations involved in dicamba OTM) and sample harvesting dates that would not likely occur in a field setting given that soybean and cotton injury following exposure to a dilute concentration of dicamba generally takes some time to induce visible symptomology (i.e. injury likely would not be present at 0 or 1 DAT to motivate sample harvest).

Objective

In order to develop a classification model capable of identifying discrete dicamba formulations present in damaged cotton and soybean tissue, research was conducted utilizing chemometric analysis of spectra obtained from FTIR analysis of soybean and cotton tissue damaged by various concentrations and formulations of dicamba at several sample timings.

Materials and Method

Design and Treatments

Experiments were conducted in 2017 and 2018 at the R.R. Foil Plant Science Research Center in Starkville, MS to develop a method for identifying dicamba formulations present in damaged soybean and cotton tissue. Experiment site information is shown in Table 2.1. The soybean cultivar 'AG4632' (ASGROW®, Bayer CropSciences, resistance to glyphosate) was seeded at 321,100 seeds ha^{-1} at a 2.5 cm depth each year. Similarly, the cotton cultivar 'DP1321' (DeltaPine®, Bayer CropSciences, resistance to glyphosate) was seeded at 119,000 seeds ha^{-1} at a 2.5 cm depth. Treatments were arranged in a six by four factorial arrangement in a randomized complete block design with inclusion of a non-treated control (NTC). Experimental units were plots consisting of four 76-cm spaced rows 12.2 m in length. The first and second rows of each

plot consisted of soybeans and the third and fourth rows consisted of cotton. Experimental factors were herbicide concentration and dicamba formulation. The six herbicide concentrations were 35, 18, 9, 4, 2, and 1 g dicamba ae ha^{-1}, which correspond to 1/32, 1/64, 1/128, 1/256, 1/512, and 1/1024 of the commonly used 1.12 kg dicamba ae ha^{-1} use rate, respectively, and fit within the range of concentrations used in previous research to simulate OTM (Egan et al. 2014; Johnson et al. 2012; Marple et al. 2007; Smith et al. 2012). Dicamba formulations were dicamba N,N-Bis-(3-Aminopropyl) Methylamine Salt ('BAPMA', Engenia®, BASF Corporation), dicamba diglycolamine salt ('DGA', Clarity®, BASF Corporation), dicamba dimethylamine salt ('DMA', Banvel®, BASF Corporation), and dicamba diglycolamine salt with potassium acetate ('DGAKAC', XTENDIMAX® herbicide with Vaporgrip® Technology, Bayer CropSciences). The molecular structure of dicamba alone and in each formulation is shown in Figure 2.1. Plots were furrow-irrigated as needed. A broadcast application of 0.87 kg ae ha^{-1} glyphosate (Roundup Powermax II™, Bayer CropSciences) was applied over the experimental area as needed to control emerging weeds; no other pesticides or fertilizers were utilized. All herbicides were applied with a carbon dioxide-pressurized plot backpack sprayer operated at 4.8 KPH and calibrated to deliver 140 L ha^{-1} at an operating pressure of 276 kPa. A four-row spray boom equipped with TTI11002 (TTI, TurboTee Induction, TeeJet Technologies, Glendale Heights, IL 60139) spray tips was operated at 51 cm above crop canopy and used to apply herbicides to the center two rows of each four row plot (one row of soybeans, one of cotton), with the outer two rows of each plot serving as a spray buffer between plots. Herbicide application occurred when cotton achieved the pinhead square growth stage (Stewart et al. 2010) and soybeans achieved the V5-V6 growth stage (Fehr and Caviness 1977). Herbicides were applied in increasing order of concentration. Spray headers were changed between each application and spray equipment was thoroughly

triple-rinsed with ammonia (WipeOut® XS, Helena Chemical Company, Collierville, TN 38017) and water between applications of each herbicide in order to avoid any cross-contamination. Latex gloves, rubber boots, and a hooded Dupont™ Tyvek® coverall spray suit (ULINE, Pleasant Prairie, WI 53158) were also utilized and changed between application of each herbicide to prevent cross-contamination of plots during herbicide application. No herbicide drift or OTM of dicamba between plots was observed in either year of research. Heat and precipitation accumulation varied by year, but growing-season totals fell within average historical ranges each year.

Data Collection and Analyses

Tissue samples were collected independently from the center two rows (one row of soybeans and one of cotton) of each plot 7, 14, 21, 28, and 56 days after treatment (DAT). Tissue samples were collected by hand-harvesting two to four visibly damaged leaves from five to seven randomly selected plants in each row for a total of 10 to 30 damaged leaves per row, per evaluation timing. Latex gloves were utilized during tissue sampling and were changed between each plot in order to avoid tissue contamination. Damaged leaves from each plot at each evaluation timing were stored together as a composite sample in a 3.78 L plastic freezer bag (Ziploc®, SC Johnson & Son, Inc., Racine, WI 53403) and transported in an ice-filled YETI® (YETI Coolers, LLC, Austin, TX 78704) cooler to the Mississippi State Chemical Laboratory, where they were stored at -80°C in a Thermo Scientific (Thermo Fisher Scientific, Waltham, MA 02451) TSC2090D chest freezer until subsequent analysis. Samples were processed by grinding the composite of frozen leaves in a mortar and pestle. Ground leaf residue was then returned to the original sample bag and stored at -80°C until spectroscopic analysis. Latex gloves were worn and changed between samples during processing, and the grinding area and equipment were

cleansed with a solution of 70% ethanol and 30% water between each composite sample. Upon conclusion of sample processing, samples were thawed to room temperature and analyzed with a Thermo Scientific (Thermo Fisher Scientific) Nicolet 6700 FTIR optical spectrometer equipped with a liquid nitrogen-cooled MCT High-D detector, KBr beamsplitter, and Smart ARK accessory (Figure 2.2). A subsample of approximately 1 g of leaf tissue from each composite sample was placed onto a ZnSe horizontal attenuated total reflectance crystal at an angle of incidence of 60°, and 10 reflections of infrared light were passed through the crystal during each scan. An infrared spectrum was generated from 64 scans of each subsample. After generation of each spectrum, the corresponding tissue subsample was disposed of, and this process was repeated four more times with subsamples from the same composite sample bag, resulting in five subsamples of each composite bag being scanned and their spectra obtained. Following analysis of each composite bag, the ZnSe crystal and Smart ARK accessory housing were sanitized utilizing a solution of 70% ethanol and 30% water. Latex gloves were worn and changed between each sample. A background spectrum of a blank sample (no plant tissue, sanitized ZnSe crystal) was collected between each composite sample to ensure no tissue residue remained between scans. A sample of approximately 5 mL of 100% polystyrene was utilized to calibrate the FTIR spectrometer periodically to ensure proper calibration. All spectra were collected at 4000 to 650 cm^{-1} frequencies and visualized using the spectral visualization software OMNIC 7.3 (Thermo Fisher Scientific). Automatic baseline and advanced attenuated total reflection (ATR) corrections were performed on each spectrum in OMNIC 7.3 prior to exporting to chemometric spectral management software for further analysis. The automatic baseline correction is commonly used to account for changes in experimental conditions during spectral measurement in order to enable accurate spectral peak and baseline identification and further

ATR correction (Cozzolino 2017). ATR correction is utilized to enable examination of samples in their original matrix (in this case leaf tissue) without further preparation required (Nunn and Nishikida 2008). Following the application of baseline and ATR correction in OMNIC 7.3, spectra were exported to the chemometric analysis software The Unscrambler X 10.5 (Camo Analytics, Magnolia, TX 77354). Spectra were then compiled by crop such that independent data matrices were constructed for soybean and cotton data. Spectra from samples treated with all concentrations and formulations and taken at all evaluation timings were pooled together. This approach allows construction of classification models that account for a wide range of concentrations, formulations, and sampling timings that would likely be present when analyzing tissue damaged by an unknown dicamba formulation, concentration, and duration since exposure, providing a practical application for the classification model. All spectra were normalized to the area under the curve in order to increase peak resolution and were subsequently converted to the first derivative utilizing the Savitzky-Golay algorithm in Unscrambler X 10.3. Derivation of the spectra with the Savitzky-Golay algorithm removes any linear baseline effects and generates the increased resolution necessary for revealing hidden spectral features that may be present in overlapping peaks. Following derivation, spectra were smoothed using Savitzky-Golay smoothing in order to reduce spectral noise.

Following pretreatment of spectral data (correction, normalization, derivation, and smoothing), dimensional reduction of the soybean and cotton data matrices was conducted via a Principal Component Analysis (PCA) run independently on soybean and cotton data matrices in The Unscrambler X 10.5. The maximum number of Principal Components (PC) for each PCA was set at seven, data were mean-centered and those with F-residuals in excess of three were removed as outliers, Hotelling's T^2 was utilized for sample leverage(Jensen and Ramirez 2017),

PCs were validated with random cross-validation of 36 samples per segment (20 segments), and the Nonlinear Iterative Partial Least Squares (NIPALS) algorithm was utilized. Following dimensional reduction, Linear Discriminant Analysis (LDA) was performed independently on each dimensionally-reduced data matrix using the linear method, and a joint PCA-LDA sample classification model was constructed in Unscrambler X 10.5. A dicamba formulation confusion matrix was generated within each LDA by utilizing a model training set of all sample spectra and a test set of randomly-selected spectra from tissue treated with each formulation. An additional PCA, LDA, and joint PCA-LDA were conducted in a similar manner to the raw spectral data in order to compare model performance before and after transforming the data with normalization and derivation.

Results and Discussion

Raw and Transformed Data Matrices

Automatic baseline- and ATR- corrected spectra (raw spectra) from cotton and soybean samples treated with the various dicamba formulations are shown pooled over dicamba concentration and sampling timing in Figures 2.3 and 2.4, respectively. The only significant peaks occurred at 3800 to 3000 cm^{-1} and 1800 to 800 cm^{-1}, however, the broad peak at 3800 to 3000 cm^{-1} is due to the O-H bend in water found in plant tissue. As such, only the spectral region commonly referred to as the 'fingerprint region' between 1800 to 800 cm^{-1} was included in spectral analysis. Raw cotton and soybean spectra narrowed to the fingerprint region between 1800 to 800 cm^{-1} from tissue treated with the various dicamba formulations are shown pooled over dicamba concentration and sampling timing in Figures 2.5 and 2.6, respectively, where an increased resolution of spectral features have become observable by narrowing the spectral focus. Normalized, derived, and smoothed cotton and soybean fingerprint spectra from tissue treated

with the various dicamba formulations are shown pooled over dicamba concentration and sampling timing in Figures 2.7 and 2.8, respectively, and reflect amplification of differences in spectral features between samples. In a preliminary analysis of similar data, Reid (2017) used PCA loading plots to determine that the most important spectral features in the soybean analyses are between 1687 and 1560 cm^{-1}. In the cotton analyses, PCA loading plot examination suggests that peaks between 1633 and 1556 cm^{-1} and 1395 to 1350 cm^{-1} are important for sample differentiation (Reid 2017). These peaks provided the basis for determining a spectral range for use in subsequent PCA and LDA analyses.

PCA, LDA, and Joint PCA-LDA on Raw Data

Cotton

PCA performed on the raw spectral data pooled across concentrations and evaluation timings resulted in the first two PC (Principal Components) accounting for 93% of the explained variance, and 99% total explained variance contained in the first 5 PC (Table 2.2). Minor sandwiching/clustering of samples by dicamba formulation can be observed in a 3D PCA graph of the first three PC (Figure 2.9). LDA of the raw spectral data pooled across concentrations and evaluation timings and using the eigenvectors generated by dimensional reduction via PCA resulted in a classification model with 39% accuracy (Table 2.3); this discrimination plot is shown in Figure 2.10, where there is evident linear clustering of samples by formulation. However, this level and pattern of clustering is significantly less structured than those reported in previous research, although said research utilized different sample media (Deng et al. 2016; Lee et al. 2009). Construction of a classification model allows the generation of a confusion matrix displaying the model's prediction of dicamba formulation in a spectrum from a given sample of crop tissue damaged by dicamba plotted against the actual value. The confusion matrix from the

classification model generated by joint LDA-PCA of raw fingerprint spectra (1800 to 800 cm^{-1}) from cotton tissue is shown in Table 2.4. The classification model performed best identifying dicamba DGAKAC (48% accuracy), and worst identifying dicamba DMA (33% accuracy). LDA conducted alone (without PCA) on the raw cotton spectral data resulted in a classification model with 85% accuracy (Table 2.3). The discrimination plot of this classification model is shown in Figure 2.13, where there is noticeable linear clustering of each formulation. The level of accuracy with this model is more in-line with previous research, although the clustering pattern remains irregular (Deng et al. 2016; Lee et al. 2009). The confusion matrix results of the classification model prediction following LDA alone is shown in Table 2.6. This classification model was most accurate when identifying dicamba BAPMA (90%) and least accurate when identifying dicamba DMA (80%) although it was no less accurate than 80% for any given formulation (Table 2.6).

Soybeans

PCA performed on the raw soybean spectral data pooled across concentrations and evaluation timings resulted in the first PC accounting for 95% of the explained variance, and 100% total explained variance contained in the first 3 PC (Table 2.2). Despite a high proportion of variance explained by PC1, the 3D score plot from the PCA on raw soybean spectral data reflects poor clustering of all formulations except dicamba BAPMA (Figure 2.14). Joint PCA-LDA of raw soybean spectral data resulted in a classification model with 35 % accuracy (Table 2.3). A discrimination plot from this model is shown in Figure 2.15, where there is some linear clustering by formulation visible, but overall clustering appears poor, again in contrast to previous work on other sample media (Deng et al. 2016; Lee et al. 2009). The resulting confusion matrix from this model is shown in Table 2.7. This classification model performed

best (48% accuracy) when classifying tissue containing dicamba DGA, and poorest (27% accuracy) when classifying tissue containing dicamba DMA (Table 2.7). When LDA was conducted alone on the raw soybean spectral data, a classification model with 80% accuracy was created (Table 2.3). A discrimination plot from this model is shown in Figure 2.18, where there is distinct linear clustering of each formulation, reflecting the model's improved classification accuracy. In this case, discrimination plot accuracy was more similar to previous work such as Ami et al. (2010), which used similar methods to classify embryonic stem cell differentiation. However, the clustering pattern was linear as opposed to the bunched patterns reported by Lehmann et al. (2015). The confusion matrix generated by the LDA alone on soybean raw spectral data is shown in Table 2.9. Accuracy of this model ranged from 76 to 84% for each of the dicamba formulations, and could thus be used to identify dicamba formulations in damaged tissue with reasonably high accuracy.

PCA and Joint PCA-LDA on Transformed Data

Cotton

PCA on cotton spectra normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and smoothed with Savitzky-Golay smoothing resulted in a 3D PC score plot shown in Figure 2.11. Poor clustering is present in this score plot, with no samples noticeably clustered by formulation (Figure 2.11). This trend is reflected in the somewhat reduced amount of total explained variation occurring in the first three PC of the PCA (65, 13, and 6%, respectively). Similarly, a joint PCA-LDA conducted on transformed cotton spectra resulted in a classification model with only 40% accuracy (Table 2.3). The poor accuracy of this model is reflected by the noticeably poor clustering of samples by formulation shown in the LDA discrimination plot in Figure 2.12, in stark contrast to the high degree of clustering shown in

previous research on other sample media (Ami et al. 2010; Deng et al. 2016; Lee et al. 2009; Lehmann et al. 2015). The poor accuracy of this classification model is further depicted in its corresponding prediction confusion matrix shown in Table 2.5. This model was only able to achieve 49% accuracy at best (when classifying samples treated with dicamba DGA) and 33% accuracy at worst (when classifying samples treated with dicamba DGAKAC, Table 2.5).

Soybeans

The 3D PC scores plot of transformed soybean spectra analyzed via PCA is shown in Figure 2.16. There is some minor clustering of samples treated with dicamba BAPMA or dicamba DMA visible, but overall clustering remains poor (Figure 2.16). Only 54% of the total explained variance in this classification is contained in PC1, with an additional 21% in PC2, and 7% in PC3, indicating poorly-clustered, highly-variable data (Table 2.2). Joint PCA-LDA of the transformed soybean spectra resulted in a classification model with 35% accuracy (Table 2.3). The discrimination plot for this model is shown in Figure 2.17, where there appears to be some linear clustering of samples by formulation, but most of which is conflated by overlapping formulation clusters (Figure 2.17). The formulation classification confusion matrix generated from this joint PCA-LDA reflects the poor clustering and accuracy of the model (Table 2.8). Accuracy of this model ranged from 32 to 38%, was best when classifying dicamba DMA, and worst when classifying dicamba BAPMA (Table 2.8). These results from transformed soybean spectra are largely similar to those of cotton in that the clustering patterns, model accuracy, and levels of explained variation are poor and dissimilar to those reported in similar research on other sample media (Ami et al. 2010; Deng et al. 2016; Lee et al. 2009; Lehmann et al. 2015).

Practical Implications for Regulatory Management

Widespread adoption of dicamba-resistant cultivars and the accompanying increase in dicamba use has resulted in a multitude of reports of dicamba OTM to susceptible crops resulting in millions of ha of crop injury (Bradley 2017; 2018). Conflating these injury cases is the potential for injury to be caused by one of many dicamba formulations, each of which affect susceptible crops similarly in terms of visible symptomology. Many of the models in this research demonstrated poor classification performance when challenged with unknown samples. However, model accuracies similar to those reported by Reid (2017) (in excess of 90%) were achieved when dicamba concentration and evaluation timing were fixed, especially at higher concentrations (data not shown). Unfortunately these models are likely not practical or realistic tools for classifying unknown samples that may be sent to the lab due to the sample bias involved in their construction (fixing concentration, etc.) which would reduce efficacy at classifying samples damaged by a vastly different concentration. As such, the models presented here based upon spectra of samples exposed to a wide range of dicamba concentrations and sampling timings are likely the best indicator of real world applicability. A counterpoint may suggest constructing several different models that each are fixed over a concentration and sampling timing and running an unknown sample through each model until adequate identification, but in a high sample-volume lab this would be both costly and inefficient.

This research shows that chemometric analyses of soybean and cotton tissue that have been damaged by various dicamba formulations and concentrations and collected at a range of evaluation timings may be useful in constructing classification models that can be used to identify the specific dicamba formulation in future unknown samples of cotton or soybean tissue, and that utilizing raw or transformed spectral data results in similar classification models. While

approaches involving PCA and joint PCA-LDA rarely achieved accuracy over 45% in the present research, utilizing LDA alone to analyze sample spectra can result in classification models capable of identifying dicamba formulations in damaged tissue with up to 90% accuracy for specific formulations, and up to 85% accuracy overall. Model accuracy varies with different formulations, but was often best when identifying dicamba DGA or dicamba DGAKAC, and worst when identifying dicamba DMA. Model accuracy also suffered when challenged with dicamba formulations that have very similar molecular structure such as dicamba DGA and dicamba DGAKAC (Figure 2.1), which is consistent with Reid et al. (2017). Construction of a more robust model that features more herbicide concentrations and evaluation timings may be possible in the future, but it appears that joint PCA-LDA modeling may not be suitable for classifying dicamba formulations in crop tissue that has been damaged by any one of many concentrations or sampled at an unknown time after exposure. LDA modeling alone appears to be a viable candidate for future applications, however. As aforementioned, models based upon a single concentration or evaluation timing, or small ranges thereof may achieve higher accuracy, but would have limited real-world applicability given the unknown nature of these variables in samples brought in to the lab for identification following an OTM event in the field. However, future research may be conducted to solve this problem by developing a technique that first uses HPLC to determine in-plant dicamba concentration in ppm, convert this concentration to an approximate herbicide rate (g ae ha^{-1}) based on leaf area of the sample, and then further analyze the sample using the appropriate classification model from the corresponding rate, which has already been shown to be accurate in previous research (Reid 2017). While such an approach may be time-consuming, it would potentially be the only method currently available for identifying dicamba formulation in damaged tissue and thus could be an important regulatory

tool in managing OTM cases. Ultimately, with further refinement, chemometric analysis of damaged crop tissue may be further developed into a cheap and efficient tool for assisting in cases of crop injury following OTM of dicamba in the future.

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Tables

Table 2.1 Table 2.1Location, year, longitude, latitude, elevation, soil type, and planting dates for experiments.[a]

Location	Year	Longitude	Latitude	Elevation	Soil Type[a]	Planting Date
				m		
Starkville	2017	88°46'W	33°27'N	84	Marietta fine sandy loam	2 May
Starkville	2018	88°46'W	33°27'N	84	Marietta fine sandy loam	1 May

[a]Source: U.S. Department of Agriculture, Natural Resources Conservation Service (2019) http://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspxtable

Table 2.2 Table 2.1Variation explained by each PC following PCA of fingerprint (1800 to 800 cm-1) spectra from cotton or soybean tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 18, 9, 4, 2, 1 g dicamba ae ha^{-1}).[a]

Data Matrix	Data Type[b]	PC1	PC2	PC3	PC4	PC5
		----------%----------				
Cotton Spectra	Raw	74	19	4	1	1
Cotton Spectra	Transformed	65	13	6	4	2
Soybean Spectra	Raw	95	3	2	-	-
Soybean Spectra	Transformed	54	21	7	4	3

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt, PC, principal component; PCA, principal component analysis

[b]Raw spectral data were not normalized, derived or smoothed; Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

Table 2.3 Table 2.2Classification model parameters following LDA alone or joint with PCA of fingerprint spectra (1800 to 800 cm-1) from cotton or soybean tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 18, 9, 4, 2, 1 g dicamba ae ha^{-1}).[a]

Data Matrix	Data Type[b]	Model Source	Accuracy
			%
Cotton Spectra	Raw	PCA-LDA	39
Cotton Spectra	Raw	LDA	84
Cotton Spectra	Transformed	PCA-LDA	39
Soybean Spectra	Raw	PCA-LDA	35
Soybean Spectra	Raw	LDA	79
Soybean Spectra	Transformed	PCA-LDA	34

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; LDA, linear discriminant analysis; PCA, principal component analysis

[b]Raw spectral data were not normalized, derived or smoothed; Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

Table 2.4 Confusion matrix from the classification model generated by LDA joint with PCA of raw fingerprint spectra (1800 to 800 cm-1) from cotton tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 18, 9, 4, 2, 1 g dicamba ae ha^{-1}).[a]

	Actual	BAPMA	DGA	DGAKAC	DMA	
Predicted		----------%----------				
BAPMA		**36**	18	19	16	
DGA		27	**40**	26	31	
DGAKAC		22	26	**48**	20	
DMA		15	16	7	**33**	
Accuracy (%)		*36*	*40*	*48*	*33*	*39*[†]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; LDA, linear discriminant analysis; PCA, principal component analysis

[†]Weighted average accuracy across all formulation classifications (overall classification model accuracy)

Table 2.5 Confusion matrix from the classification model generated by LDA joint with PCA of transformed fingerprint (1800 to 800 cm-1) from cotton tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 18, 9, 4, 2, 1 g dicamba ae ha^{-1}).[a,b]

	Actual	BAPMA	DGA	DGAKAC	DMA	
Predicted		----------%----------				
BAPMA		**42**	15	29	21	
DGA		25	**49**	28	32	
DGAKAC		23	24	**33**	11	
DMA		10	13	10	**35**	
Accuracy (%)		*42*	*49*	*33*	*35*	*40*[†]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; LDA, linear discriminant analysis; PCA, principal component analysis

[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

[†]Weighted average accuracy across all formulation classifications (overall classification model accuracy)

Table 2.6 Confusion matrix from the classification model generated by LDA without PCA of raw fingerprint spectra (1800 to 800 cm-1) of cotton tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 18, 9, 4, 2, 1 g dicamba ae ha^{-1}).[a]

	Actual	BAPMA	DGA	DGAKAC	DMA	
Predicted		----------%----------				
BAPMA		**90**	4	5	4	
DGA		1	**84**	5	11	
DGAKAC		6	4	**85**	5	
DMA		3	8	5	**80**	
Accuracy (%)		*90*	*84*	*85*	*80*	*85*[†]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; LDA, linear discriminant analysis; PCA, principal component analysis

[†]Weighted average accuracy across all formulation classifications (overall classification model accuracy)

Table 2.7 Confusion matrix from the classification model generated by LDA joint with PCA of raw fingerprint spectra (1800 to 800 cm-1) from soybean tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 18, 9, 4, 2, 1 g dicamba ae ha^{-1}).[a]

	Actual	BAPMA	DGA	DGAKAC	DMA	
Predicted		----------%----------				
BAPMA		**32**	20	13	15	
DGA		40	**47**	40	38	
DGAKAC		15	18	**33**	20	
DMA		13	15	14	**27**	
Accuracy (%)		*32*	*47*	*33*	*27*	*35*[†]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; LDA, linear discriminant analysis; PCA, principal component analysis

[†]Weighted average accuracy across all formulation classifications (overall classification model accuracy)

Table 2.8 Confusion matrix from the classification model generated by LDA joint with PCA of transformed fingerprint (1800 to 800 cm-1) from soybean tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 18, 9, 4, 2, 1 g dicamba ae ha^{-1}).[a,b]

	Actual	BAPMA	DGA	DGAKAC	DMA	
Predicted		----------%----------				
BAPMA		**32**	29	24	22	
DGA		25	**33**	23	27	
DGAKAC		24	19	**36**	13	
DMA		19	19	17	**38**	
Accuracy (%)		*32*	*33*	*36*	*38*	*34*[†]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; LDA, linear discriminant analysis; PCA, principal component analysis

[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

[†]Weighted average accuracy across all formulation classifications (overall classification model accuracy)

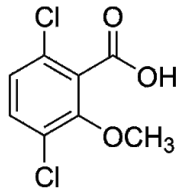
Table 2.9 Confusion matrix from the classification model generated by LDA without PCA of raw fingerprint spectra (1800 to 800 cm-1) of soybean tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 18, 9, 4, 2, 1 g dicamba ae ha^{-1}).[a]

	Actual	BAPMA	DGA	DGAKAC	DMA	
Predicted		----------%----------				
BAPMA		**79**	1	3	4	
DGA		11	**84**	11	17	
DGAKAC		3	7	**80**	3	
DMA		7	8	6	**76**	
Accuracy (%)		*79*	*84*	*80*	*76*	*80*[†]

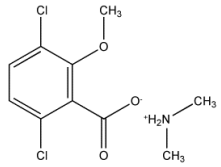
[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; LDA, linear discriminant analysis; PCA, principal component analysis

[†]Weighted average accuracy across all formulation classifications (overall classification model accuracy)

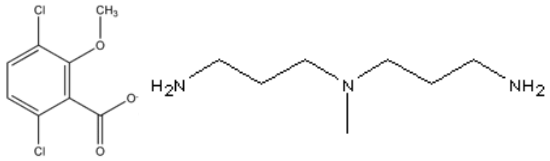
Figures



Dicamba Acid



Dimethylamine salt of dicamba



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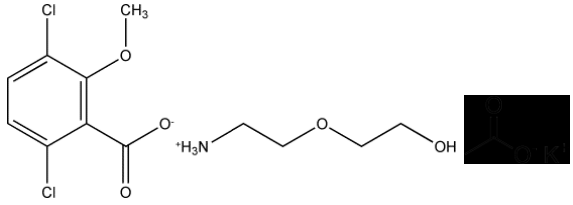
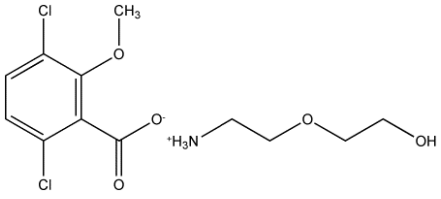


Diglycolamine salt of dicamba with potassium acetate



Diglycolamine salt of dicamba

Figure 2.1 Chemical structure of dicamba (3,6-dichloro-2-methoxybenzoic acid) alone and formulated with DGA, DMA, DGAKAC or BAPMA.[a]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt

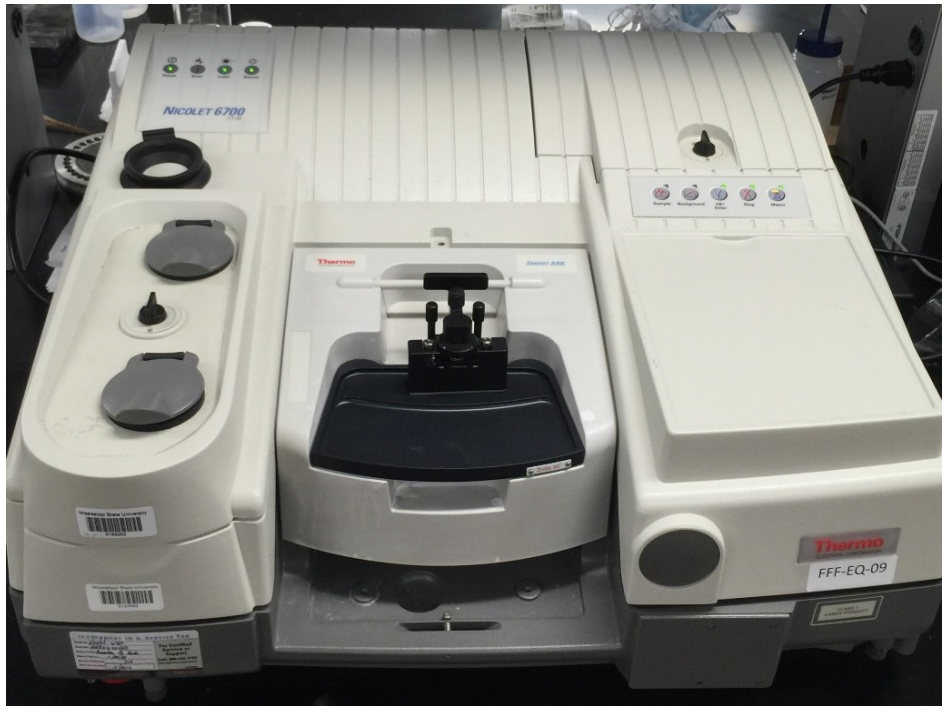


Figure 2.2 Thermo Nicolet 6700 FTIR optical spectrometer equipped with a liquid nitrogen-cooled MCT High-D detector, KBr beamsplitter, and Smart ARK accessory with a ZnSe horizontal attenuated total reflectance crystal at a 60° angle of incidence.

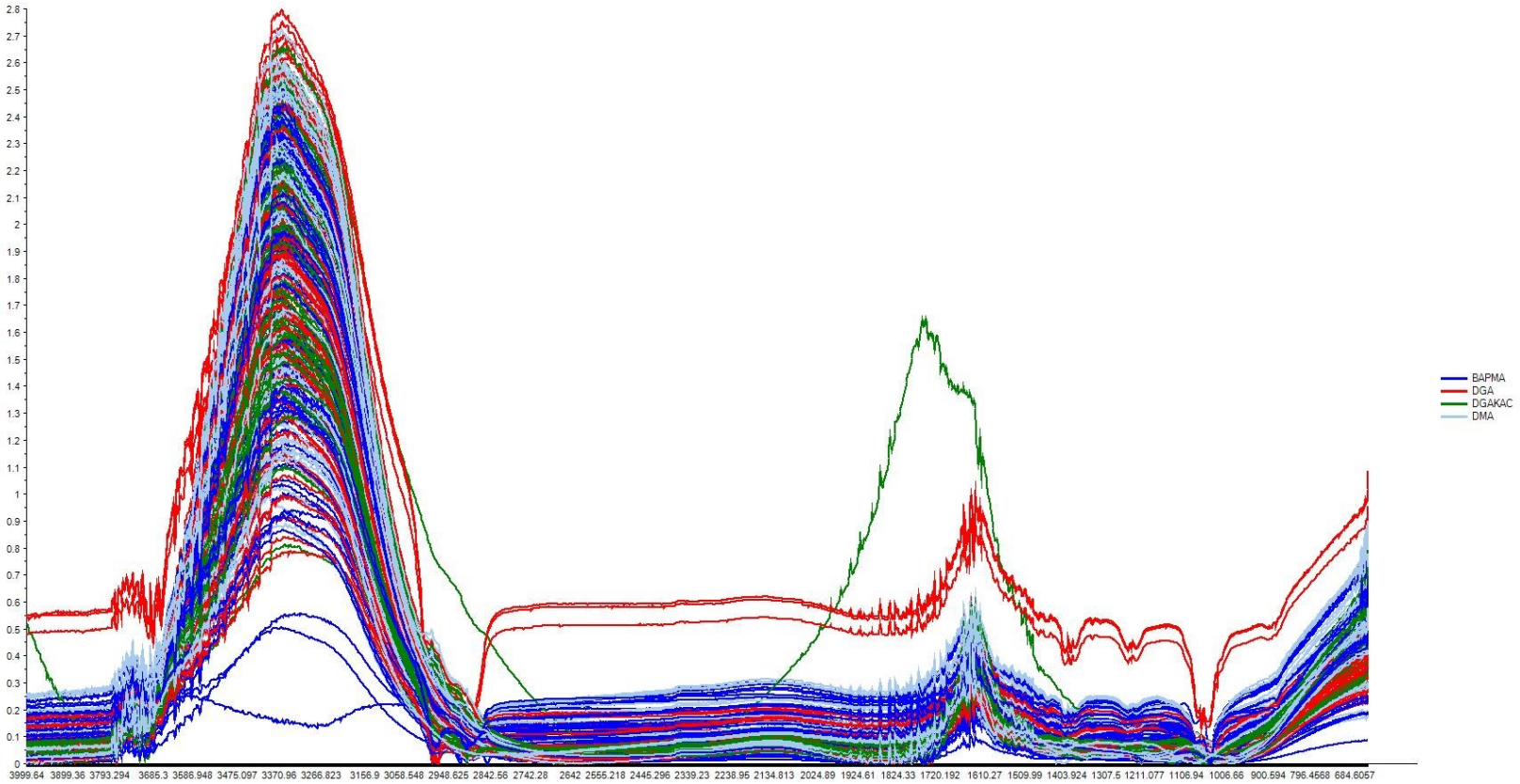


Figure 2.3 Raw spectra (4000 to 650 cm^{-1}) from cotton tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a,b]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt

[b]Y-axis: proportion of infrared light absorbed by the sample (%); X-axis infrared frequency (cm^{-1})

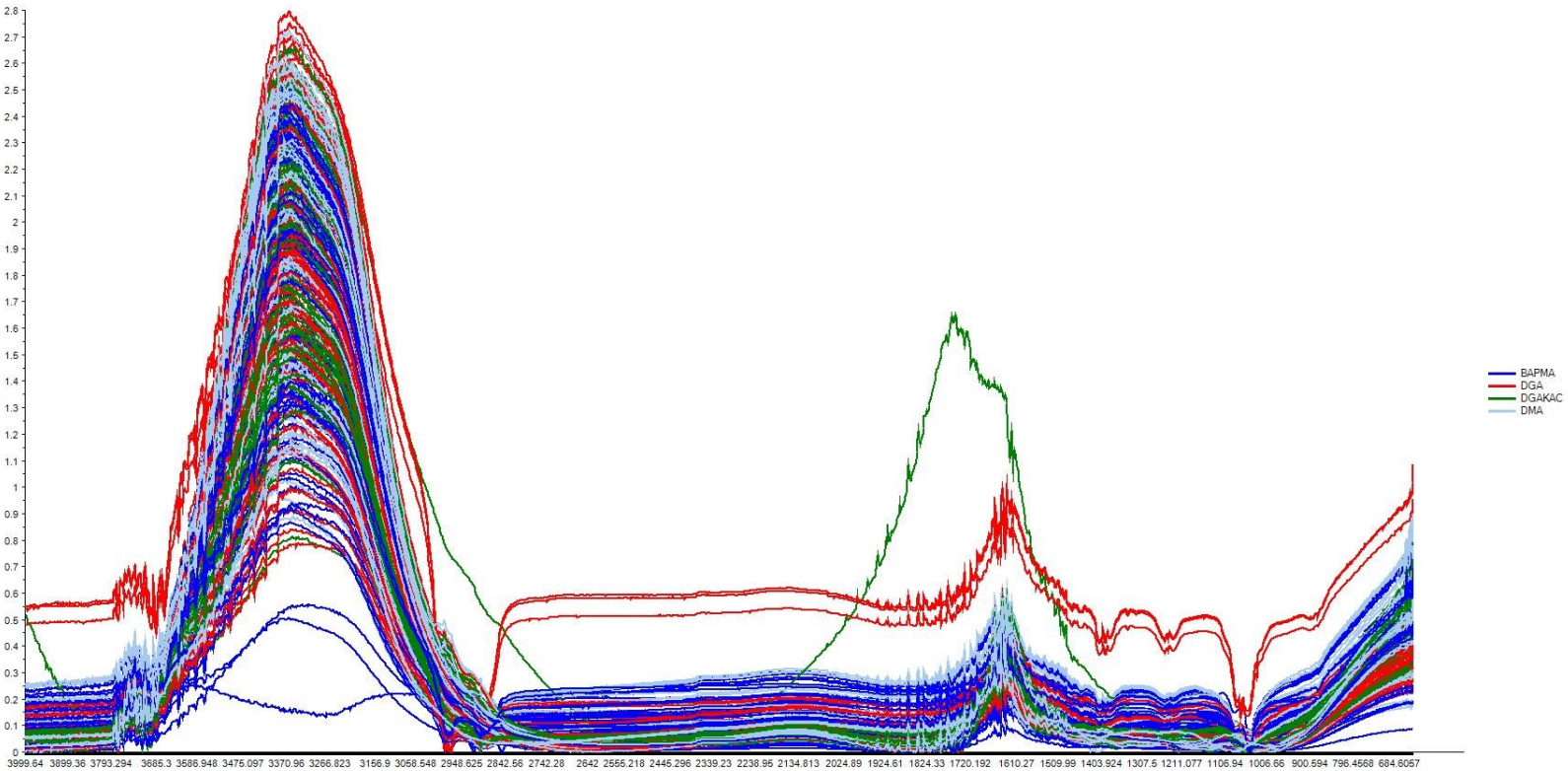


Figure 2.4 Raw spectra (4000 to 650 cm^{-1}) from soybean tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a,b]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt

[b]Y-axis: proportion of infrared light absorbed by the sample (%); X-axis infrared frequency (cm^{-1})

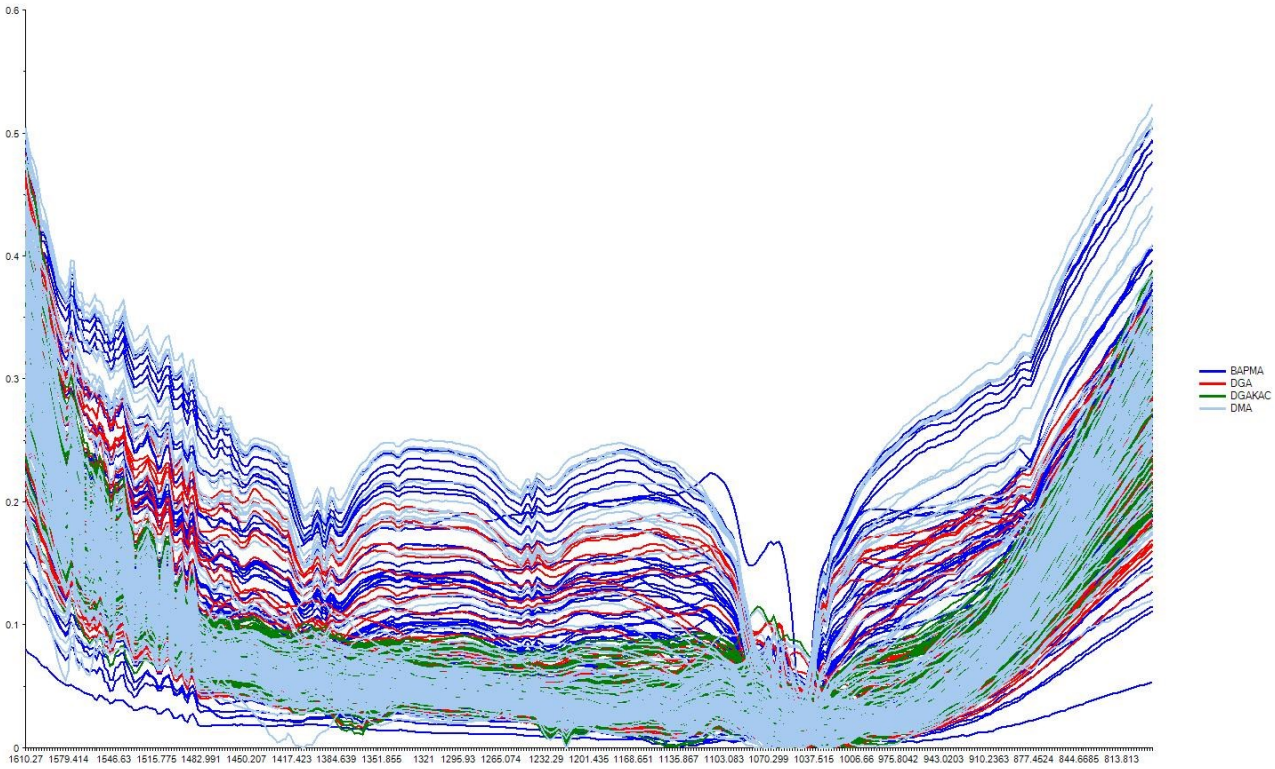


Figure 2.5 Raw fingerprint spectra (1800 to 800 cm^{-1}) from cotton tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a,b]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt

[b]Y-axis: proportion of infrared light absorbed by the sample (%); X-axis infrared frequency (cm^{-1})

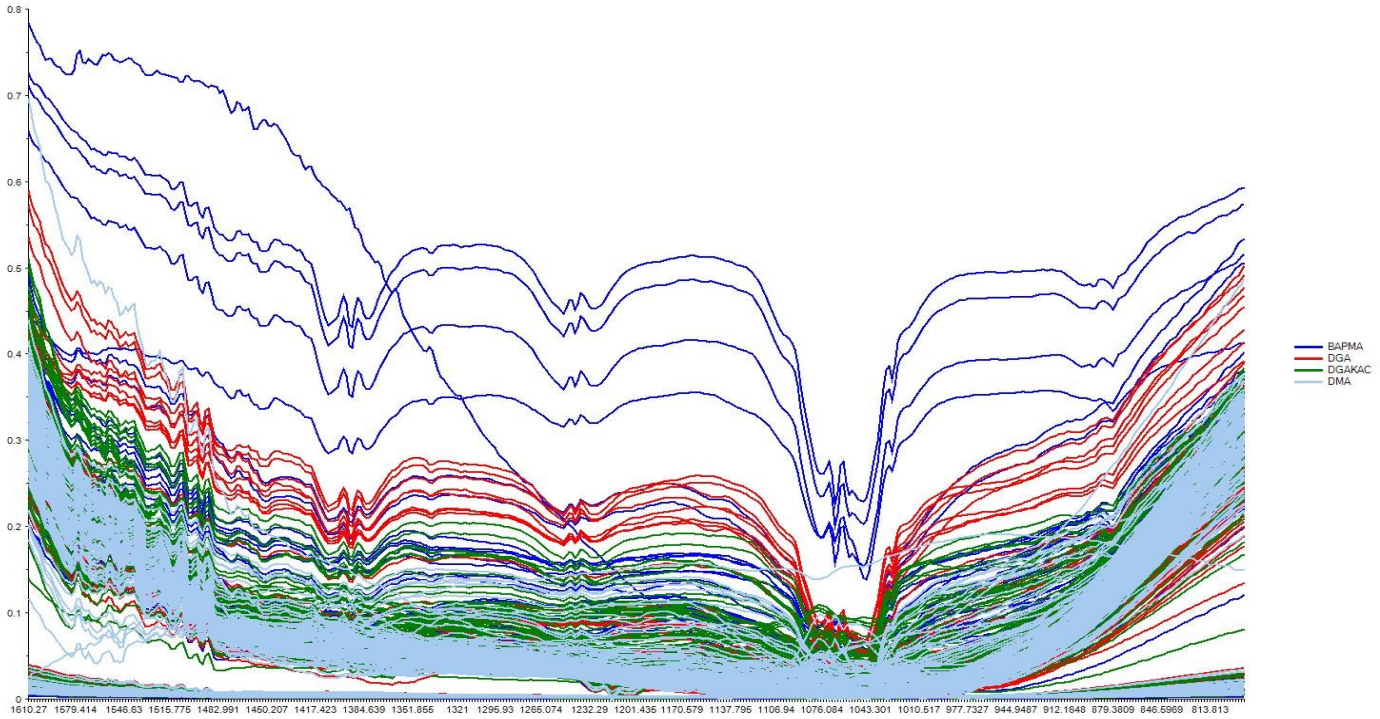


Figure 2.6 Raw fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a,b]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt

[b]Y-axis: proportion of infrared light absorbed by the sample (%); X-axis infrared frequency (cm^{-1})

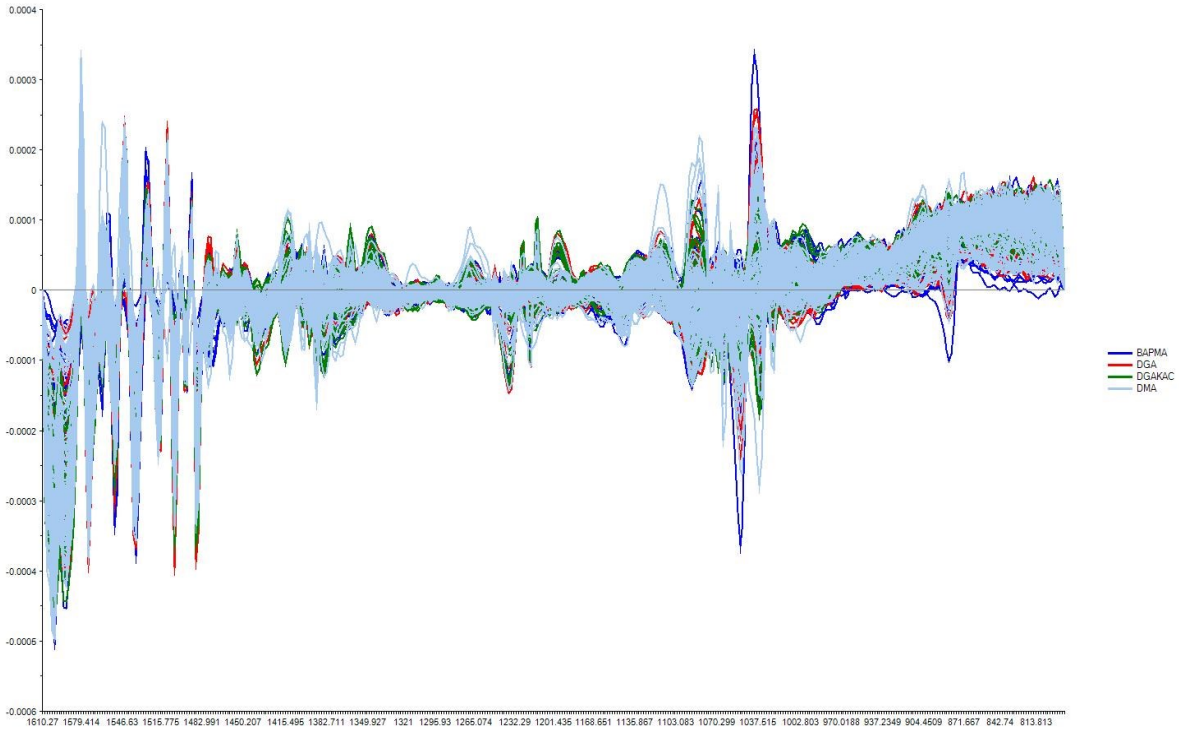


Figure 2.7 Transformed fingerprint spectra (1800 to 800 cm^{-1}) from cotton tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a,b,c]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt

[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

[c]Y-axis: proportion of infrared light absorbed by the sample (%); X-axis infrared frequency (cm^{-1})

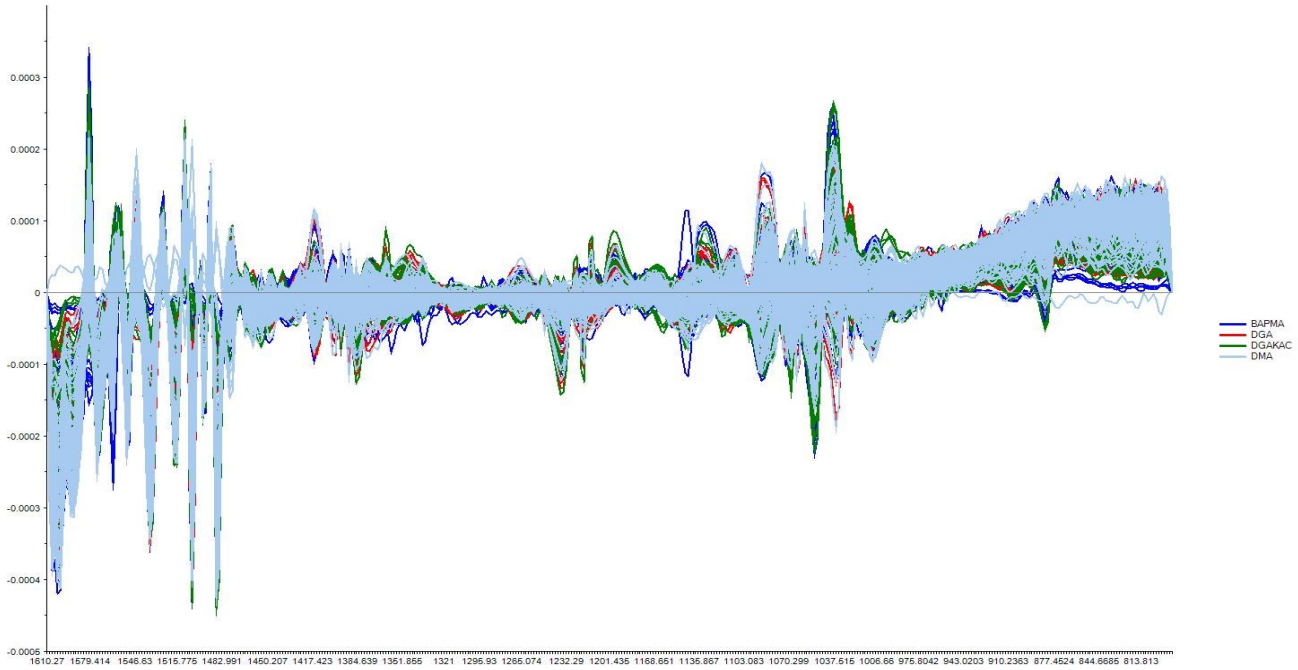


Figure 2.8 Transformed fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a,b,c]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt

[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

[c]Y-axis: proportion of infrared light absorbed by the sample (%); X-axis infrared frequency (cm^{-1})

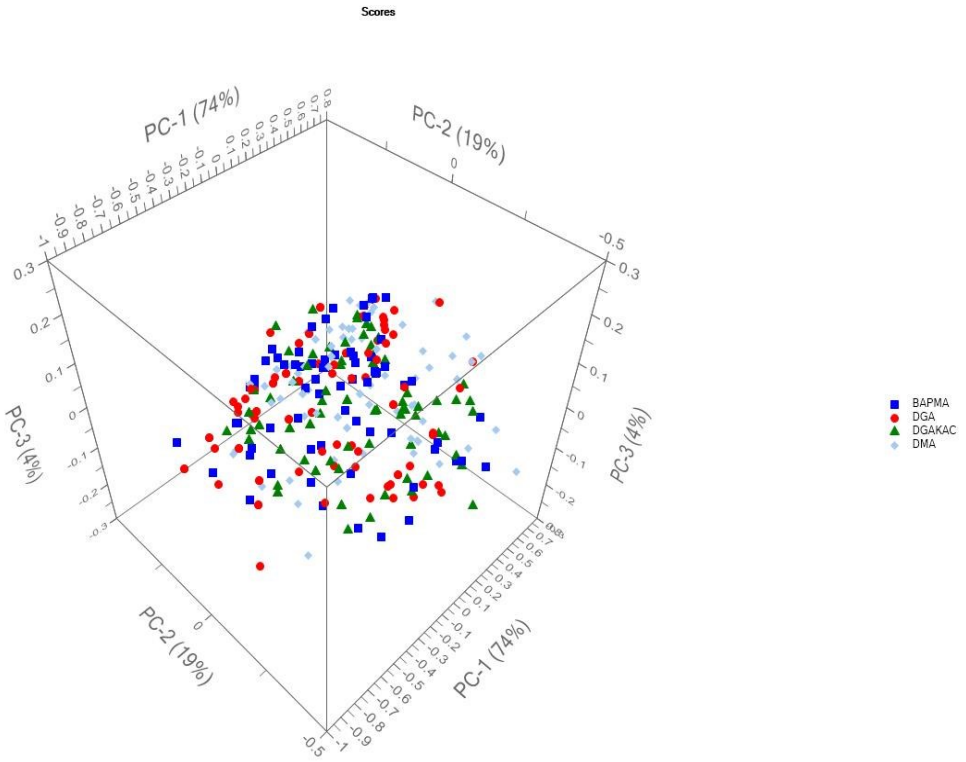


Figure 2.9 3D PCA score plot of raw fingerprint spectra (1800 to 800 cm^{-1}) from cotton tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; PCA, principal component analysis

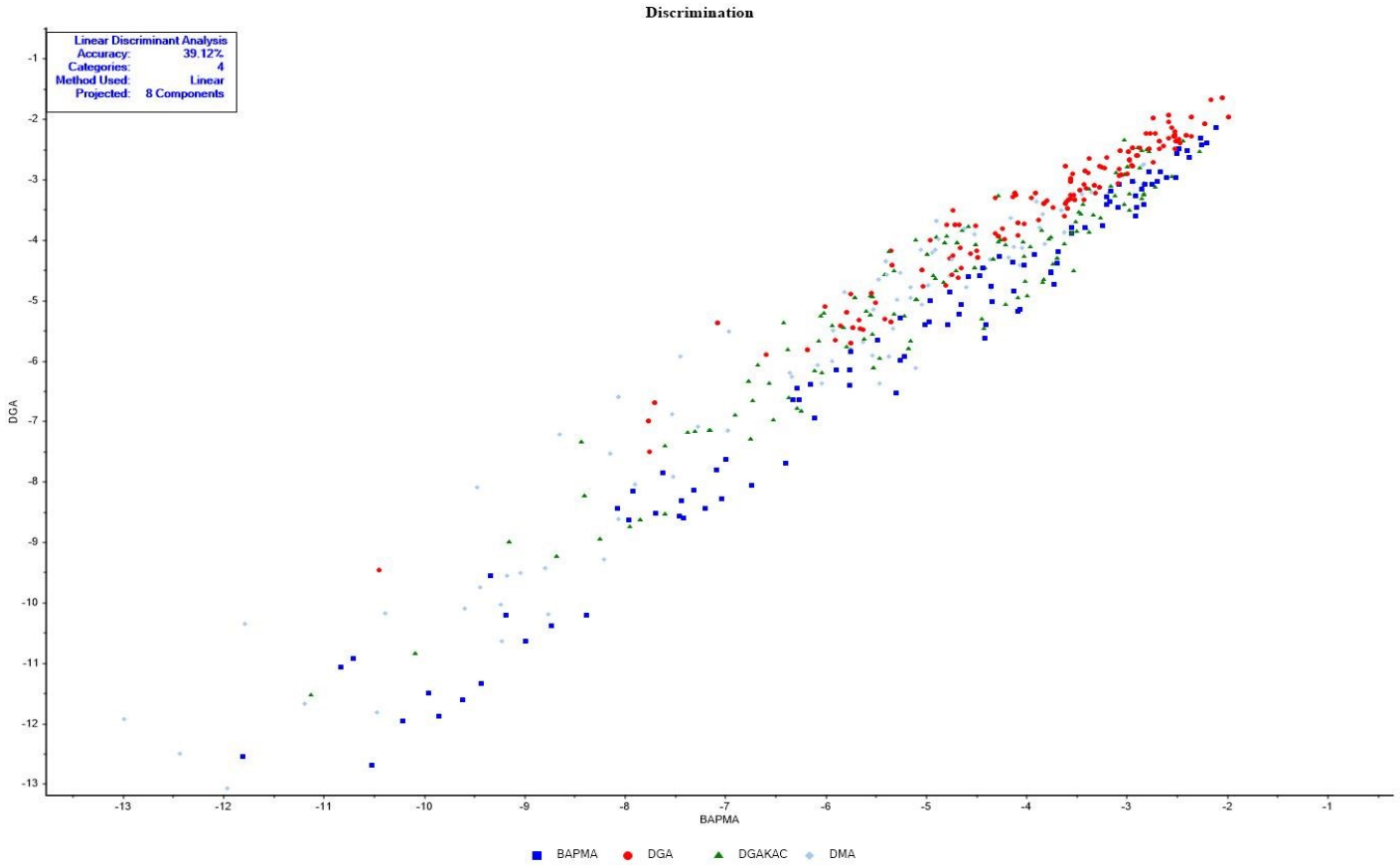


Figure 2.10 LDA discrimination plot following PCA of raw fingerprint spectra (1800 to 800 cm^{-1}) from cotton tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; LDA, linear discriminant analysis; PCA, principal component analysis

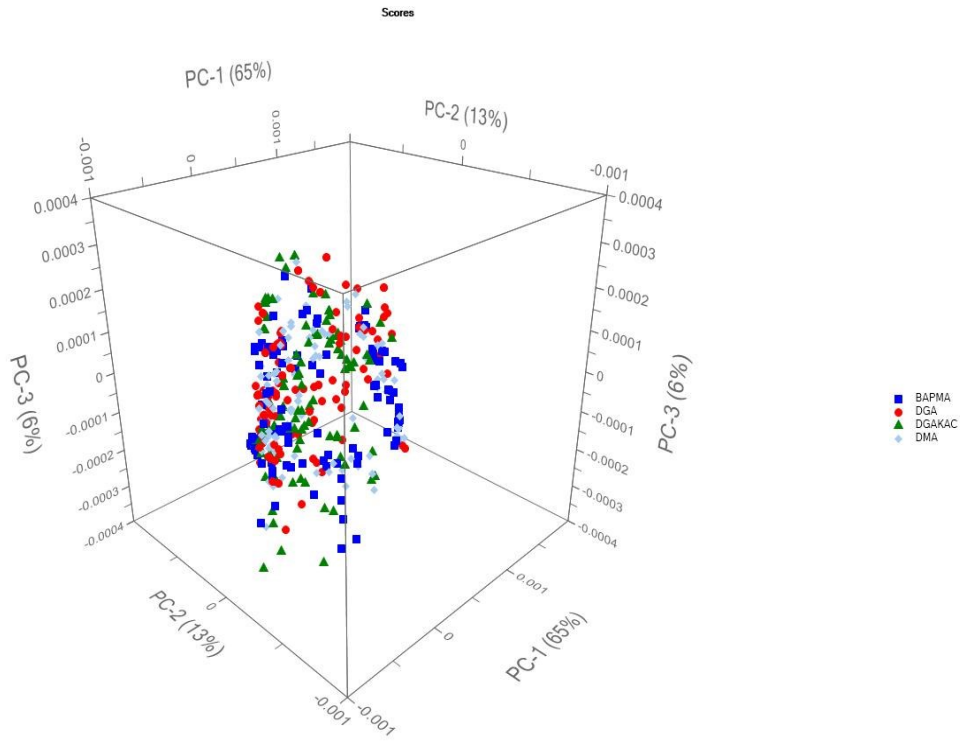


Figure 2.11 3D PCA score plot of transformed fingerprint spectra (1800 to 800 cm^{-1}) from cotton tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a,b]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; PCA, principal component analysis

[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

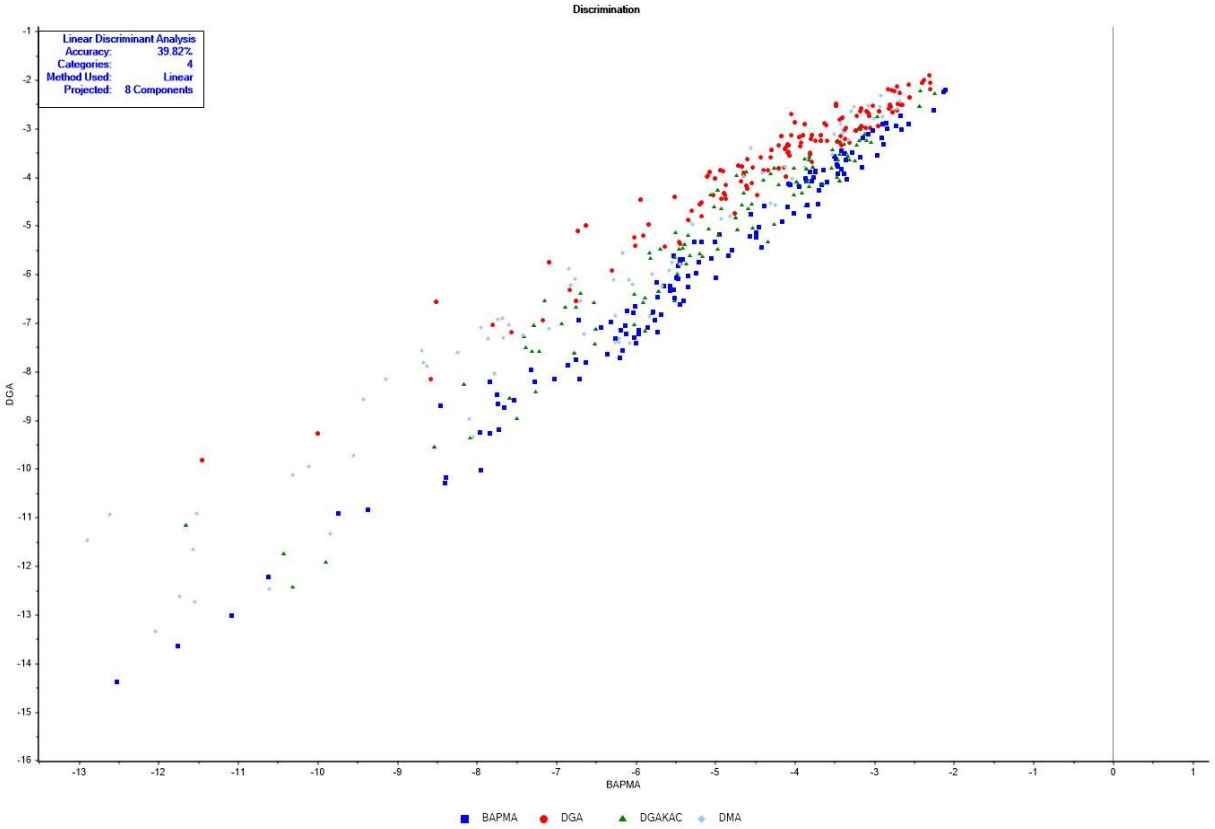


Figure 2.12 LDA discrimination plot following PCA of transformed fingerprint spectra (1800 to 800 cm^{-1}) from cotton tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a,b]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; LDA, linear discriminant analysis; PCA, principal component analysis

[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

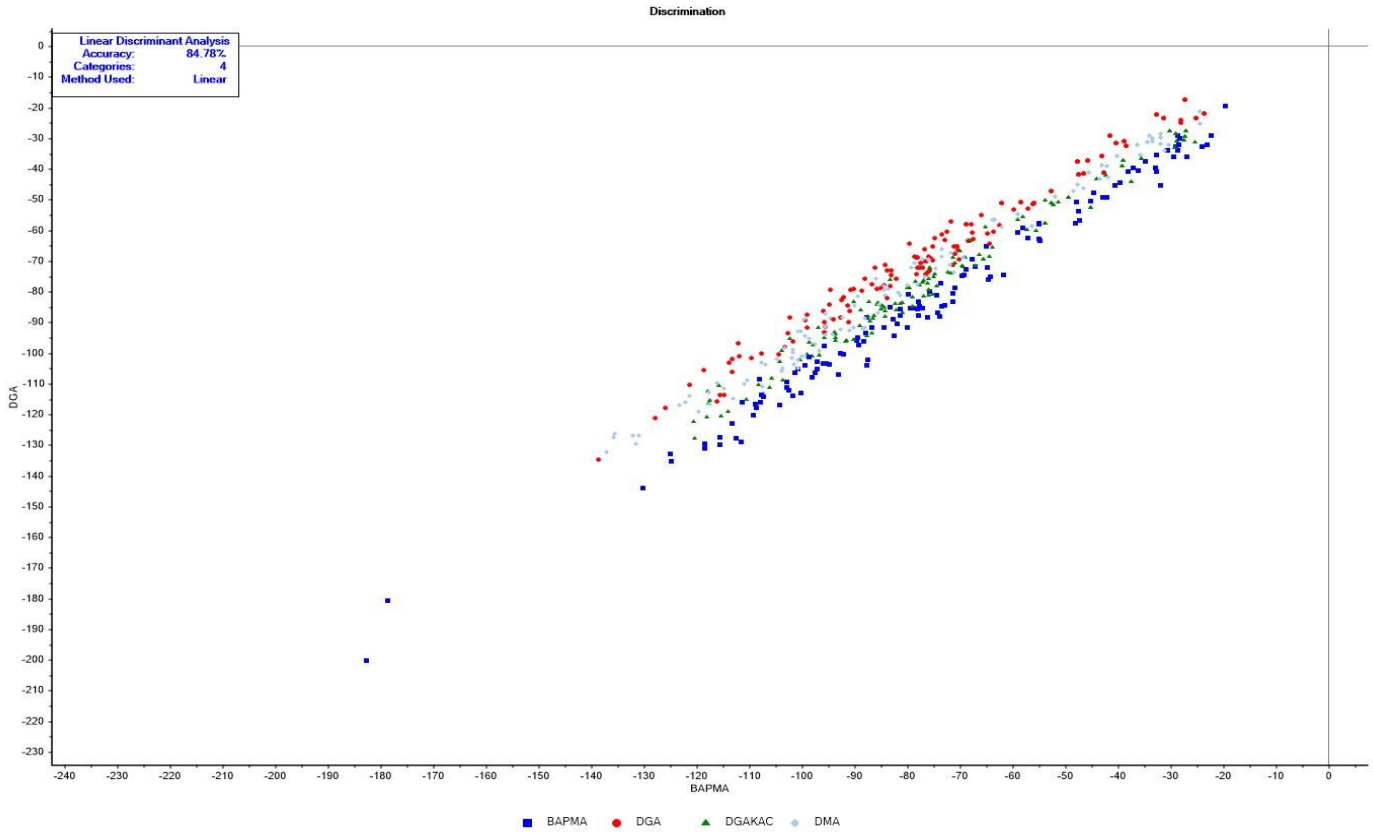


Figure 2.13 LDA discrimination plot without PCA of raw fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; LDA, linear discriminant analysis; PCA, principal component analysis

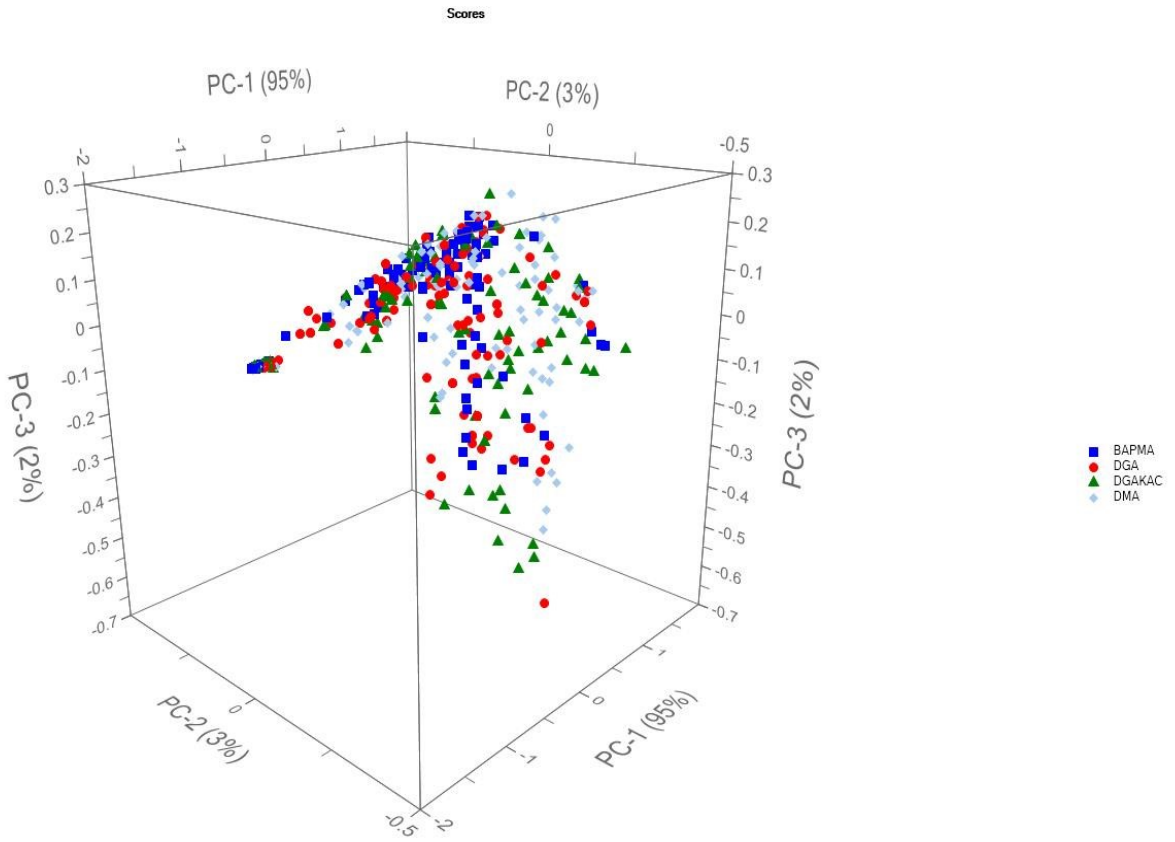


Figure 2.14 3D PCA score plot of raw fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; PCA, principal component analysis

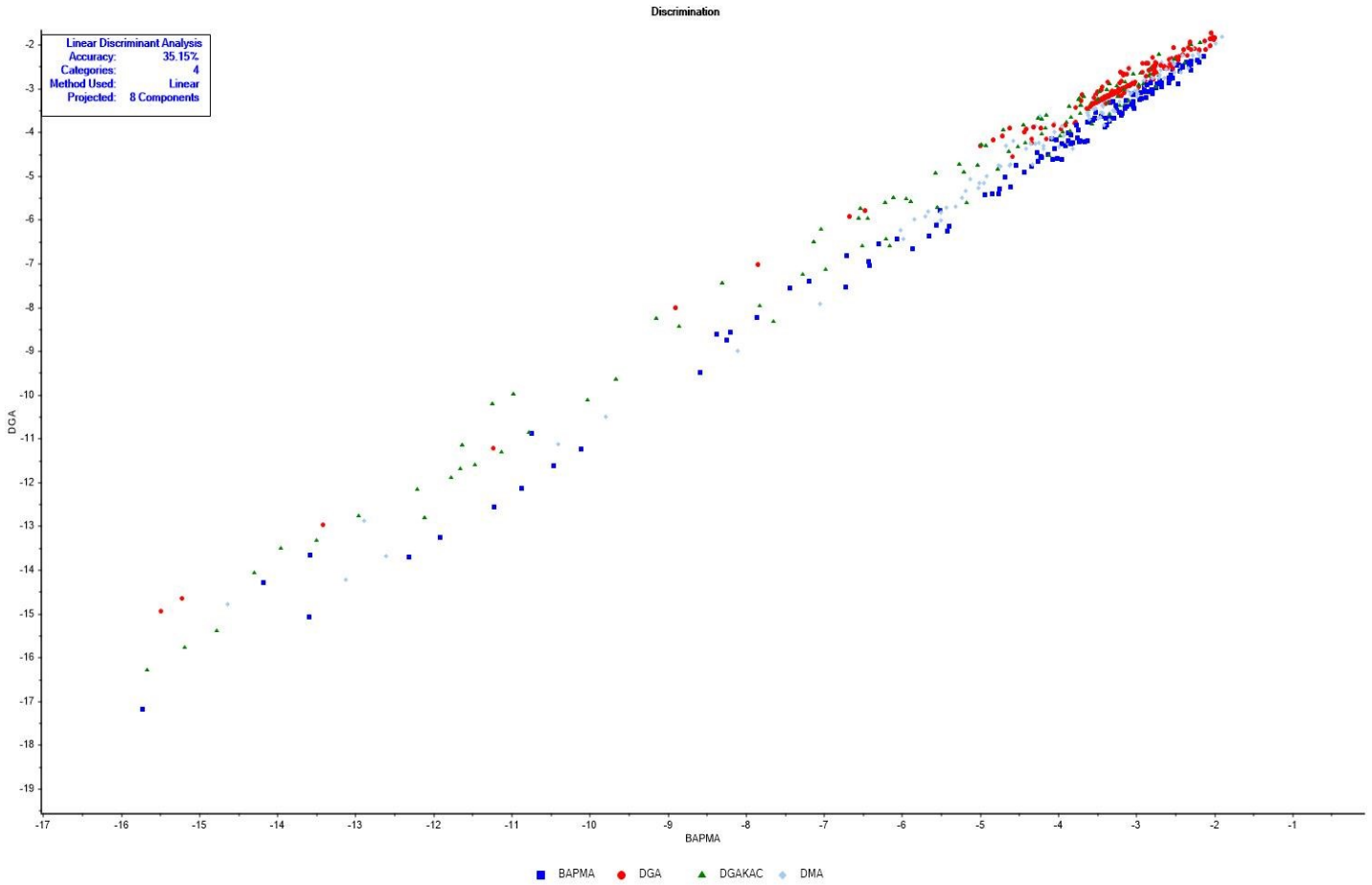


Figure 2.15 LDA discrimination plot following PCA of raw fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; LDA, linear discriminant analysis; PCA, principal component analysis

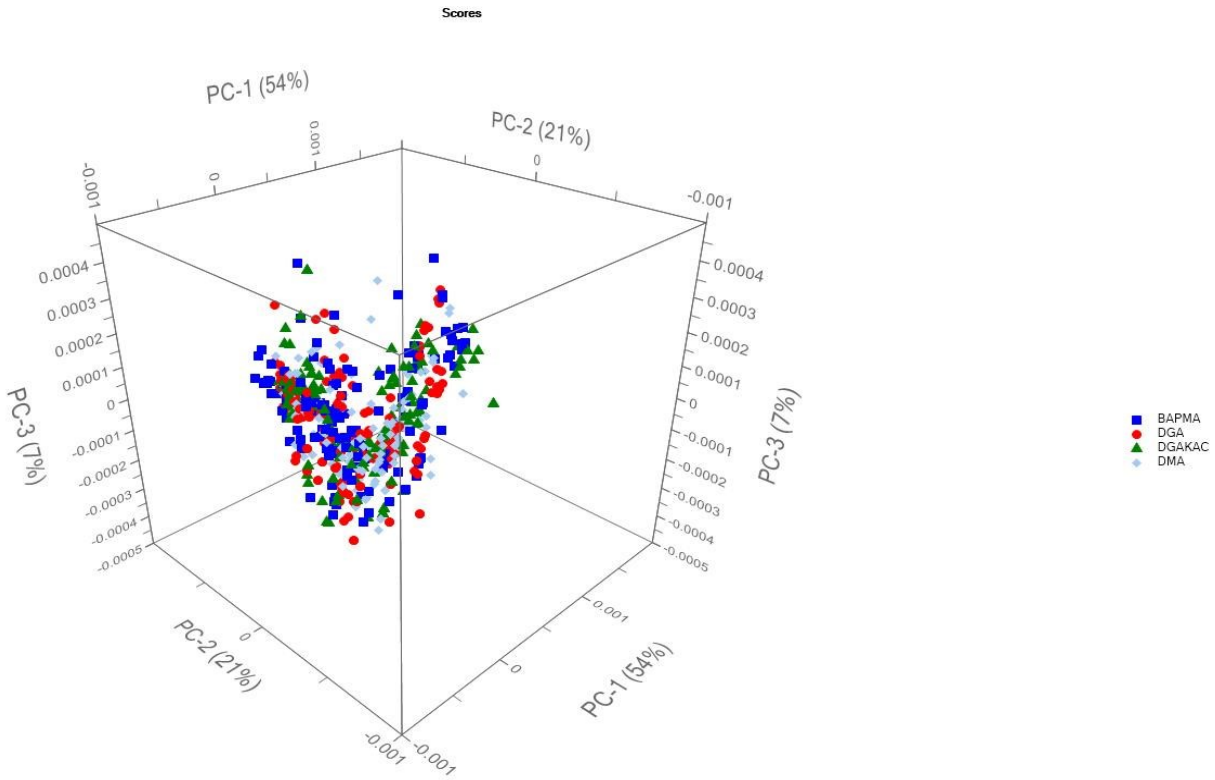


Figure 2.16 3D PCA score plot of transformed fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a,b]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; PCA, principal component analysis

[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

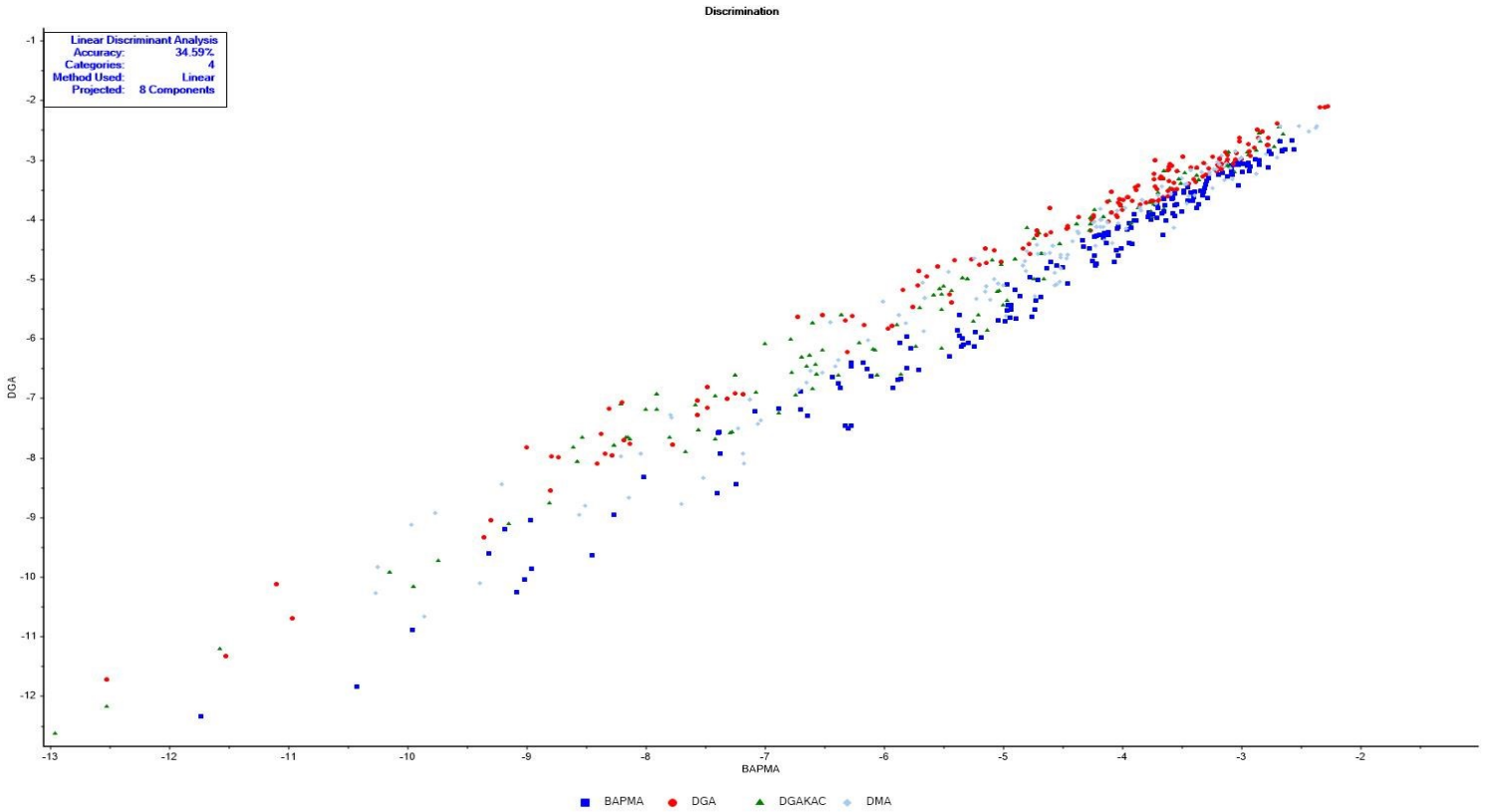


Figure 2.17 LDA discrimination plot following PCA of transformed fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a,b]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; LDA, linear discriminant analysis; PCA, principal component analysis

[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

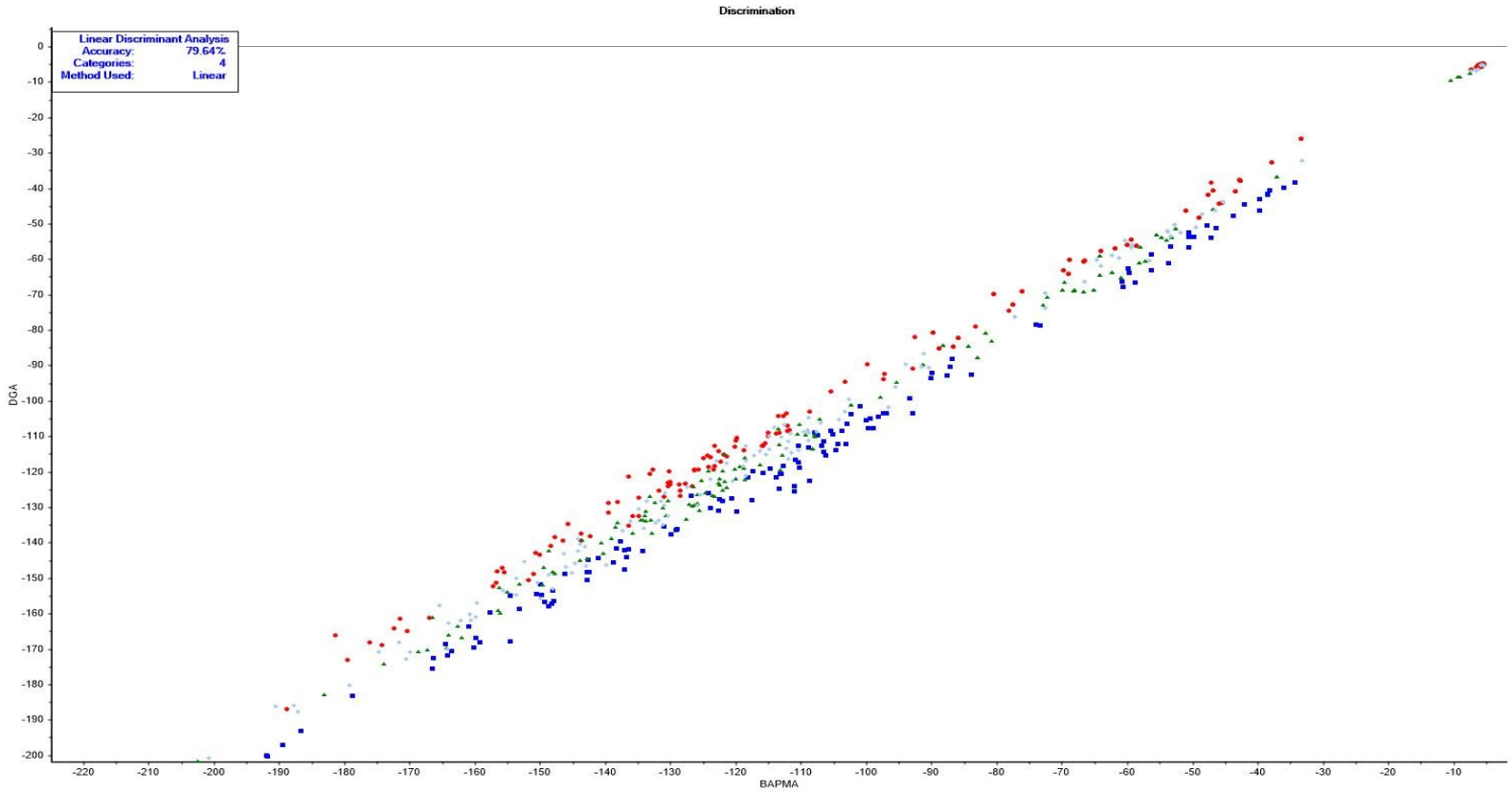


Figure 2.18 LDA discrimination plot without PCA of raw fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with dicamba DGA, dicamba DMA, dicamba DGAKAC or dicamba BAPMA, pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and dicamba concentration (35, 17.5, 8.75, 4.375, 2.1875, 1.09375 g dicamba ae ha^{-1}).[a]

[a]Abbreviations: BAPMA, N,N-Bis-(3-Aminopropyl) methylamine salt; DGA, diglycolamine salt; DGAKAC, diglycolamine salt with potassium acetate; DMA, dimethylamine salt; LDA, linear discriminant analysis; PCA, principal component analysis

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CHAPTER III

APPLICATION OF FTIR SPECTROSCOPY AND CHEMOMETRICS FOR THE CLASSIFICATION OF 2,4-D FORMULATIONS IN DAMAGED COTTON AND SOYBEAN TISSUE

Abstract

Increased use of 2,4-D in row crop production may lead to increased cases of damage to susceptible cotton and soybeans following off-target movement (OTM) of 2,4-D. Research was conducted in 2017 and 2018 in Starkville, MS to develop a method using chemometrics and spectroscopy to produce classification models capable of identifying specific 2,4-D formulations present in damaged crop tissue. 2,4-D acid (ACID), dimethylamine salt (DMA), choline salt (CHOLINE), and isooctyl ester (ESTER) were applied to susceptible cotton and soybeans at 33, 17, 8, 4, 2, and 1 g 2,4-D ae ha^{-1}, and samples were analyzed via infrared spectroscopy to generate spectra which were then analyzed by principal component analysis (PCA) and linear discriminant analysis (LDA). Joint PCA-LDA models were only capable of classifying 2,4-D formulation in damaged tissue with up to 36% accuracy, whereas LDA alone produced models with 77 to 80% accuracy. Models performed worst when classifying 2,4-D DMA or ESTER and best when classifying 2,4-D CHOLINE or ACID. Model accuracies were similar regardless of sample media (soybean or cotton tissue) or data format (raw spectral data vs normalized, derived, and smoothed spectra). This research suggests that with further refining, chemometric analysis of

spectral data from damaged crop tissue may be an economical, efficient, and promising application to support management of crop injury following OTM of 2,4-D.

Nomenclature: 2,4-D; acid; choline; cotton, *Gossypium hirsutum* L.; dimethylamine; isooctyl ester; soybean, *Glycine max* L. [Merr.]

Key words: 2,4-D, chemometrics, cotton, formulation, off-target movement, soybeans

Introduction

Increased use of auxin herbicides following commercialization of new weed control technologies such as the 2,4-D-based Enlist™ Weed Control System (Corteva Agriscience, Indianapolis, IN 46268) has added a tool for controlling herbicide-resistant (HR) weeds such as Palmer amaranth (*Amaranthus palmeri* S. Watson), but has not come without challenges. Adoption of these technologies has been widespread, with up to 60% of soybean and cotton ha in some areas being planted to dicamba-resistant cultivars alone (Lingenfelter et al. 2017; USDA 2017). Producers have adopted these technologies in hopes to access elite crop germplasm, protect their crops from off-target movement (OTM) of auxin herbicides, and have the ability to use auxin herbicides POST, especially given the relatively low incidence of 2,4-D-resistant weeds (Heap et al. 2019; Egan et al. 2014; Mortensen et al. 2012). However, due to the highly efficacious nature of auxin herbicides such as 2,4-D and its mode-of-action (MOA) as a synthetic plant hormone, exposure of a susceptible species such as non-target soybeans or cotton to even a sub-lethal concentration of 2,4-D can cause severe crop injury (Johnson et al. 2012; Marple et al. 2007, 2008; Sciumbato et al. 2004; Staten 1946). Susceptible crops can be exposed to OTM of 2,4-D and other auxin herbicides in many ways including herbicide spray drift, spray equipment contamination, herbicide volatility and vapor drift, and temperature inversions following application to a target area (Boerboom 2004; Cundiff et al. 2017; Egan et al. 2014; Mortensen et

al. 2012). Increased use of auxin herbicides following the introduction of new weed control technologies has resulted in several reports of damaged crops in the U.S. In 2017, over 1.4 million ha of soybeans were damaged by OTM of dicamba, and a further 440,000 ha of soybeans were injured by dicamba by July 2018 (Bradley 2017, 2018).

The issue of auxin herbicide OTM and subsequent crop injury is exacerbated by the fact that while only products containing the choline salt formulation of 2,4-D (Enlist Duo® or Enlist One™ herbicide with Colex-D® technology, Corteva Agriscience) are labeled for use in the Enlist™ Weed Control System, there are several other 2,4-D-containing products available for use in other applications such as cereal and grain crops, range and pasture, or rights-of-way. Non-choline salt formulations of 2,4-D that are available and commonly used include 2,4-D dimethylamine, 2,4-D acid, and 2,4-D isooctyl ester. This consideration renders management of 2,4-D OTM cases more nuanced given the potential for crop injury being the result of: (1) OTM of a legally-applied choline salt formulation, (2) OTM of a non-choline salt formulation applied illegally to Enlist™ cultivars, (3) OTM of a non-choline salt formulation legally applied to a nearby area. Producers may be motivated to illegally apply older, non-choline salt 2,4-D formulations that exhibit higher volatility and proclivity for OTM to Enlist™ cultivars for many reasons such as cheaper herbicide costs (Egan et al. 2014; Mortensen et al. 2012). However, managing cases of 2,4-D OTM damaging susceptible soybeans in cotton is complicated given that, generally, crop response and visible symptomology are very similar (leaf malformation, stem and petiole epinasty, callus tissue formation, node and ht effects, necrosis) regardless of the 2,4-D formulation that caused the injury (Byrd et al. 2015; Egan et al. 2014; Johnson et al. 2012; Marple et al. 2007; Sciumbato et al. 2004). Similar crop response following exposure to each 2,4-D formulation makes it easy to conflate the 2,4-D formulations that may have caused injury,

especially at the field observation level. Previous research into differences in crop response to auxin herbicides have reported differential crop response following exposure to the same auxin herbicide in different formulations, depending on crop and herbicide. Thompson et al. (2007) reported 7% less soybean injury following exposure to 2,4-D amine relative to 2,4-D ester. Bauerle et al. (2015) found increased tomato (*Solanum lycopersicum* L.) sensitivity to 2,4-D ester relative to 2,4-D amine or acid. Similarly, Sosnoskie et al. (2014) observed reduced cotton ht and increased injury following exposure to 2,4-D ester relative to 2,4-D amine or choline. In tomato and sunflower (*Helianthus annus* L.) treated with various formulations of triclopyr, Dias et al. (2017) found increased injury from triclopyr trimethylamine relative to triclopyr butoxyethyl ester, triclopyr pyridinyloxyacetic acid, or triclopyr choline; and increased injury following exposure to triclopyr trimethylamine or triclopyr choline relative to other triclopyr formulations tested in soybean.

Unfortunately, cotton and soybean response to different 2,4-D formulations does vary by formulation, but only at a small magnitude of difference which is difficult to detect by observation at the field level (Buol et al. 2019). Accordingly, there is need for an alternative method to identify specific 2,4-D formulations present in damaged crop tissue in order to better manage cases of 2,4-D OTM. Several analytic techniques are available for analyzing agricultural samples and identifying herbicidal compounds in various media. High-performance liquid chromatography/mass spectrometry (HPLC/MS), gas chromatography/mass spectrometry (GC/MS), and inductively coupled plasma optical emission spectrometry (ICP-OES), among others, have all been used for detecting and identifying low herbicide concentrations. Gavlick et al. (2015) developed a method with HPLC/MS to compare volatility of 2,4-D formulations in plastic reservoirs. Peña et al. (2002) utilized GC/MS to identify phenylureas herbicides in plant

tissue, and Duke et al. (2018) used ICP-OES to detect and identify mineral and amino acid content in soybean seeds. Unfortunately, such techniques are not feasible for identifying 2,4-D formulations in damaged crop tissue due to their requirement of sample preparation methods that raise pH by adding sodium hydroxide, a process that cleaves the functional groups of the formulation molecule (dimethylamine salt, choline salt, isooctyl ester) that are required for 2,4-D formulation identification (Reid 2017). However, an alternative method that is also cheaper and faster is Fourier-Transform infrared spectroscopy (FTIR). FTIR is advantageous in that it is fast, economical, and does not require much, if any, sample preparation, allowing a high volume of ground, unaltered plant tissue samples to be analyzed in a relatively short period of time. FTIR spectroscopy measures the interaction between infrared radiation and the sample media (Simonescu 2012). Measuring the pattern and magnitude of infrared light passed through or absorbed by a sample allows inferences to be made about the chemical content of the sample. Absorbance spectra produced by FTIR plot relative absorbance by frequency in reciprocal centimeters (cm^{-1}, 'wavenumbers'), which are reciprocally related to wavelength. IR absorbance spectra to provide insight into the composition of a sample (Simonescu 2012). This technique has been utilized to detect and identify dilute amounts of various analytes in a wide range of media. Puckrin et al. (1996) characterized gas concentrations in air samples taken from the troposphere using FTIR. This technique is bolstered when combined with statistical analyses such as Principal Component Analysis (PCA), Linear Discriminant Analysis (LDA), or joint PCA-LDA to build classification models from spectral data in a process called chemometrics. PCA is a modeling technique that describes sample-variable relationships via dimensional reduction, a process that transforms data into a new coordinate system with a condensed number of variables (dimensions) called Principal Components (PC, also called eigenvectors). PCs are

linear combinations of the original variables and are constructed such that they are orthogonal to each other. Generating PCs creates a new data dimension vector that captures as much variation in the original dataset as possible. Several PCs are generated such that the first PC captures the greatest amount of variation, the second PC captures the second most, and so on. The total amount of variation captured by PCs is the total explained variation considered by the classification model. LDA is a supervised classification method used to classify unknown sample based on a set of known sample classifications used to create a model. Joint PCA-LDA results in a model that utilizes the dimension vectors produced by PCA to consider the within and between group variation in a dataset and apply these parameters towards predicting unknown sample classification (Ami et al. 2010; Reid 2017). Reid (2017) reported preliminary PCA-LDA classification models capable of up to 95% accuracy in classifying 2,4-D formulations (acid, choline salt, dimethylamine salt, isooctyl ester) in damaged cotton tissue harvested 0, 1, 3, 7, 21, and 28 DAT that had been treated with 8 g 2,4-D ae ha^{-1}. While these models are promising, they only considered one relatively high (in an OTM context) 2,4-D concentration and sample harvesting dates that would be unlikely in a field setting given that soybean and cotton injury following exposure to a dilute concentration of 2,4-D can take time to induce visible symptomology (i.e. injury likely would not be present at 0 or 1 DAT).

Objective

In order to develop a classification model capable of identifying specific 2,4-D formulation present in damaged cotton and soybean tissue, research was conducted utilizing chemometric analysis of spectra obtained from FTIR spectroscopy conducted on soybean and cotton tissue damaged by various concentrations and formulations of 2,4-D at several sample timings.

Materials and Method

Design and Treatments

Research was conducted in 2017 and 2018 at the R.R. Foil Plant Science Research Center in Starkville, MS to develop a method for identifying formulated 2,4-D in damaged soybean and cotton tissue, and site information is shown in Table 3.1. Cotton cultivar 'DP1321' (DeltaPine®, Bayer CropScience, resistance to glyphosate) was seeded at 119,000 seeds ha^{-1}, and soybean variety 'AG4632' (ASGROW®, Bayer CropScience, resistance to glyphosate) was seeded at 321,100 seeds ha^{-1} at a 2.5 cm depth each year. Treatments were arranged in a six by four factorial arrangement in a randomized complete block design with a non-treated control (NTC). Experimental units were plots consisting of four 76-cm spaced rows 12.2 m in length. The first and second rows of each plot consisted of cotton, and the third and fourth rows consisted of soybeans. Experimental factors were herbicide concentration and 2,4-D formulation. The six herbicide concentrations were 33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}, which correspond to 1/32, 1/64, 1/128, 1/256, 1/512, and 1/1024 of the commonly used 1.07 kg 2,4-D ae ha^{-1} use rate, respectively. These concentrations fit within the range used in previous research to simulate OTM of 2,4-D (Byrd et al. 2015; Egan et al. 2014; Everitt and Keeling 2009; Johnson et al. 2012; Scholtes et al. 2014). The four 2,4-D formulations tested included 2,4-D acid ('ACID', Unison® Novel Broadleaf, Helena Chemical Company, Collierville, TN 38017), 2,4-D choline ('CHOLINE', Enlist One™ herbicide with Colex-D® technology, Corteva Agriscience), 2,4-D dimethylamine ('DMA', Weedar® 64, Nufarm Agricultural Products, Alsip, IL 60803), and 2,4-D isooctyl ester ('ESTER', Weedone® LV4 EC, Nufarm Agricultural Products). The molecular structure of 2,4-D alone and in each formulation is shown in Figure 3.1. Plots were furrow-irrigated as needed throughout the growing season. A broadcast application of 0.87 kg ae ha^{-1}

glyphosate (Roundup Powermax II™, Bayer CropScience) was broadcast applied as needed to control emerging weeds and no other pesticides or fertilizers were utilized. Herbicide applications were made using a carbon dioxide-pressurized plot backpack sprayer operated at 4.8 KPH calibrated to deliver 140 L ha^{-1} at 276 kPa. A four-row spray boom with TTI11002 (TTI, TurboTee Induction, TeeJet Technologies, Glendale Heights, IL 60139) spray tips was operated 51 cm above crop canopy to apply herbicides to the center two rows of each plot, with the outer two rows serving as a spray buffer between plots. Herbicide application was conducted when cotton achieved the pinhead square growth stage (Stewart et al. 2010) and soybeans achieved the V5-V6 growth stage (Fehr and Caviness 1977). Herbicides were applied in increasing order of concentration using. New spray headers were used for each application and spray equipment was thoroughly triple-rinsed with ammonia (WipeOut® XS, Helena Chemical Company) and water between applications of each herbicide in order to prevent cross-contamination. Latex gloves, rubber boots, and a hooded Dupont™ Tyvek® coverall spray suit (ULINE, Pleasant Prairie, WI 53158) were changed between application of each herbicide to further prevent cross-contamination of plots. No herbicide drift or OTM between plots was observed in either year of research. Heat and precipitation accumulation varied by year but fell within average historical ranges each year.

Data Collection and Analyses

Tissue samples were collected independently by crop from each of the center two rows of each plot 7, 14, 21, 28, and 56 days after treatment (DAT). Tissue samples were collected by hand-harvesting two to four visibly damaged leaves from five to seven randomly selected plants in each row for a total of 10 to 30 damaged leaves per crop, per evaluation timing. Latex gloves were worn during tissue sampling and changed between each plot in order to avoid tissue

contamination. Leaves from each plot were stored together as a composite sample in a 3.78 L plastic freezer bag (Ziploc®, SC Johnson & Son, Inc., Racine, WI 53403) and transported in an ice-filled YETI® (YETI Coolers, LLC, Austin, TX 78704) cooler to the Mississippi State Chemical Laboratory, where they were stored at -80°C in a Thermo Scientific (Thermo Fisher Scientific, Waltham, MA 02451) TSC2090D chest freezer. Samples were processed by grinding each composite in a mortar and pestle. Latex gloves were changed between samples during processing and the grinding area and equipment were cleansed with a solution of 70% ethanol and 30% water between each sample. After sample processing, samples were thawed to room temperature and analyzed with a Thermo Scientific (Thermo Fisher Scientific) Nicolet 6700 FTIR optical spectrometer equipped with a liquid nitrogen-cooled MCT High-D detector, KBr beamsplitter, and Smart ARK accessory (Figure 3.2). A subsample of approximately 1 g of leaf tissue from each composite sample was placed on a ZnSe horizontal attenuated total reflectance crystal at an angle of incidence of 60°, and 10 reflections of infrared light were passed through the crystal per scan. Infrared spectra were generated from 64 scans of each subsample. After obtaining each subsample spectrum, the corresponding tissue subsample was disposed of and this process was repeated four more times with additional subsamples from the same composite sample bag, resulting in five subsamples of each composite bag being scanned. Following analysis of each composite bag, the ZnSe crystal and Smart ARK accessory housing were sanitized utilizing a solution of 70% ethanol and 30% water. Latex gloves were changed between each sample. A background spectrum of a blank sample (no plant tissue, sanitized ZnSe crystal) was collected between each composite sample to ensure no cross-contamination. A sample of approximately 5 mL of 100% polystyrene was used to calibrate the spectrometer periodically to ensure instrument accuracy. All spectra were collected from 4000 to 650 cm^{-1} and visualized

using the software OMNIC 7.3 (Thermo Fisher Scientific). Automatic baseline and advanced attenuated total reflection (ATR) corrections were performed on each spectrum in OMNIC 7.3 before exporting to chemometric spectral management software. Automatic baseline correction is commonly used to account for changes in experimental conditions during spectral measurement such as temperature shifts in order to enable accurate spectral peak and baseline identification (Cozzolino 2017). ATR correction is utilized to enable the examination of samples in their original matrix without further preparation (Nunn and Nishikida 2008). Following baseline and ATR correction in OMNIC 7.3, spectra were exported to the chemometric analysis software The Unscrambler X 10.5 (Camo Analytics, Magnolia, TX 77354). Spectra were compiled by crop such that independent data matrices were constructed for soybean and cotton data. Spectra from samples treated with all concentrations and formulations and taken at all evaluation timings were pooled. Utilizing a pooled approach is in contrast to Reid (2017), but is more practical by allowing the construction of classification models that account for a wide range of concentrations, formulations, and sampling timings that would likely be present when analyzing tissue samples damaged by an unknown 2,4-D formulation, concentration, and duration since exposure. Spectra were normalized to the area under the curve in order to increase peak resolution, and converted to the first derivative utilizing the Savitzky-Golay algorithm in Unscrambler X 10.3. Derivation of the spectra via the Savitzky-Golay algorithm removes any linear baseline effects and generates the increased resolution necessary to reveal hidden spectral features conflated in overlapping peaks. Spectra were also smoothed using Savitzky-Golay smoothing in order to reduce noise.

Following pretreatment of spectral data (correction, normalization, derivation, and smoothing), dimensional reduction of data matrices was conducted with PCA run independently

on soybean and cotton data matrices in The Unscrambler X 10.5. The maximum number of PC for each PCA was set at seven. PCA data were mean-centered and those with F-residuals in excess of three were removed as outliers. Hotelling's T^2 was utilized for sample leverage (Jensen and Ramirez 2017) and PCs were validated with random cross-validation of 36 samples per segment (20 segments). The Nonlinear Iterative Partial Least Squares (NIPALS) algorithm was utilized in construction of the PCA model. Following PCA, LDA was performed using the linear method (the quadratic and mahalanobis methods resulted in the poorest classification), and a joint PCA-LDA sample classification model was constructed. 2,4-D formulation confusion matrices were generated within each LDA by utilizing a model training set of all sample spectra and a test set of randomly-selected spectra from tissue treated with each formulation. An additional PCA, LDA, and joint PCA-LDA were similarly conducted on raw spectral data in order to compare model performance before and after transforming the data with normalization and derivation.

Results and Discussion

Raw and Transformed Data Matrices

Baseline- and ATR- corrected spectra (raw spectra) from cotton and soybean samples treated with the different 2,4-D formulations are shown pooled over 2,4-D concentration and sampling timing in Figures 3.3 and 3.4, respectively. Significant peaks occurred at 3800 to 3000 cm^{-1} and 1800 to 800 cm^{-1}. The broad peak at 3800 to 3000 cm^{-1} is due to the O-H bend in water found in plant tissue and was ignored in further analysis. The spectral region commonly referred to as the 'fingerprint region' between 1800 to 800 cm^{-1} was included in spectral analysis. Raw cotton and soybean fingerprint spectra from tissue treated with various 2,4-D formulations are shown pooled over concentration and sampling timing in Figures 3.5 and 3.6, respectively. Increased

resolution of spectral features became observable by narrowing the spectral focus in these Figures. Normalized, derived, and smoothed cotton and soybean fingerprint spectra are shown pooled over concentration and sampling timing in Figures 3.7 and 3.8, respectively, and depict amplified differences in spectral features between samples. In a preliminary analysis of similar data, Reid (2017) used PCA loading plots to identify the most important spectral features in the soybean analyses at 1687 and 1560 cm^{-1}. Similarly, PCA loading plot examination determined peaks between 1633 and 1556 cm^{-1} and 1395 to 1350 cm^{-1} are important for soybean sample classification(Reid 2017). These spectral features provided the basis for determining a spectral range for use in subsequent PCA and LDA.

PCA, LDA, and Joint PCA-LDA on Raw Data

Cotton

PCA performed on the raw data pooled across concentrations and evaluation timings resulted in PC1 and PC2 accounting for 89 and 7% of the explained variation, respectively, and 100% total explained variation contained in the first 4 PC (Table 3.2). A 3D PCA scores plot of the first three PC demonstrates little clustering by 2,4-D formulation, despite the high amount of variation contained in the first three PC (Figure 3.9). LDA of the raw data pooled across concentrations and evaluation timings and using the eigenvectors generated by dimensional reduction via PCA produced a classification model with 33% accuracy (Table 3.3). The discrimination plot for this model is shown in Figure 3.10, where there is some linearization of samples by formulation. This linear pattern of the discrimination plot is in contrast to the distinct clustering by formulation reported by Reid (2017) and other previous research in different media (Deng et al. 2016; Lee et al. 2009; Lehmann et al. 2015). Lack of distinct clustering is likely due to the nature of these models as classifying formulation across multiple concentrations and

evaluation timings. The corresponding confusion matrix displaying the PCA-LDA model's prediction of 2,4-D formulation from a given sample of crop tissue plotted against the actual value is shown in Table 3.4. The classification model performed best identifying 2,4-D CHOLINE (44% accuracy), and worst identifying 2,4-D DMA (18% accuracy). LDA conducted alone (without PCA) on the raw cotton spectra produced a classification model with 77% accuracy, a significant improvement over the joint PCA-LDA model (Table 3.3). The discrimination plot of this model is shown in Figure 3.13, where there is noticeable linear clustering of each formulation. The level of accuracy produced by this model is more consistent with previous research, although the clustering pattern remains irregular (Deng et al. 2016; Lee et al. 2009; Lehmann et al. 2015; Reid 2017). The corresponding confusion matrix following LDA alone is shown in Table 3.6 which demonstrates the most accuracy when identifying 2,4-D CHOLINE (89%) and least accurate when identifying 2,4-D DMA (71%) although even the poorest accuracy of this model (71%, Table 3.6) was a significant improvement over the joint PCA-LDA model and is closer to the accuracy reported by Reid (2017).

Soybeans

PCA performed on the raw soybean spectral data across concentrations and evaluation timings generated five PCs accounting for 69, 23, 5, 1, and 1% variation in PCs 1, 2, 3, 4, and 5, respectively, for a total of 99% variation contained in the first 5 PCs (Table 3.2). Despite a high proportion of variation explained by PC1 and PC2, the 3D score plot again reflects poor clustering by formulation (Figure 3.14). Joint PCA-LDA of resulted in a classification model with 36% accuracy (Table 3.3) and the discrimination plot for this model is shown in Figure 3.15. Linearization of samples by variation is present but overall clustering remains poor relative to previous work in other sample media (Deng et al. 2016; Lee et al. 2009). The corresponding

confusion matrix from this model is shown in Table 3.7. This model was most accurate (44%, 45% accuracy) when classifying tissue containing 2,4-D CHOLINE and ACID (respectively), and least accurate (16% accuracy) when classifying tissue containing 2,4-D ESTER (Table 3.7), which is in contrast to the cotton models that were most accurate classifying 2,4-D CHOLINE and least accurate with 2,4-D DMA. LDA conducted alone on the raw soybean spectral data produced a classification model with 80% accuracy (Table 3.3). Figure 3.18 shows a discrimination plot from this model with distinct linear clustering of each formulation. The accuracy of this model was more similar to Reid (2017) although the clustering pattern was linear as opposed to the bunched patterns reported by previous research (Reid 2017; Lehmann et al. 2015), likely due to analysis over a wide range of variable levels (concentrations, sample timings), as opposed to the fixed levels found in most previous research. This LDA's corresponding confusion matrix is shown in Table 3.9. Up to 80 to 81% accuracy was achieved by this model when classifying 2,4-D CHOLINE and 2,4-D DMA, respectively, and the poorest accuracy was still reasonable (66%) when classifying 2,4-D ESTER (Table 3.9).

PCA and PCA-LDA on Transformed Data

Cotton

PCA of cotton spectra normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and smoothed with Savitzky-Golay smoothing produced a 3D PC score plot shown in Figure 3.11, which does not reflect distinct clustering by formulation despite the first two PCs accounting for 71 and 12% variation, respectively. Joint PCA-LDA on transformed cotton spectra constructed a classification model with only 35% accuracy (Table 3.3), which is reflected by the lack of clustering by formulation in the LDA discrimination plot (Figure 3.12). In contrast, Reid (2017) produced discrimination plots with a high degree of

clustering, but only utilized one concentration fixed over sampling timing. The corresponding confusion matrix for the classification model produced by PCA-LDA on transformed cotton spectra is presented in Table 3.5. This model was able to achieve up to 45% accuracy when classifying samples treated with 2,4-D CHOLINE and 29% accuracy when classifying samples treated with 2,4-D DMA, the same trend in classification performance observed in models from analysis of non-transformed (raw) cotton spectra.

Soybeans

A 3D PC scores plot of transformed soybean spectra is shown in Figure 3.16 which depicts little distinct clustering by formulation, despite 83% of the total explained variation being contained in PC1 (Table 3.2). Joint PCA-LDA produced a classification model with 32% overall accuracy (Table 3.3). The discrimination plot for this model depicts linearization of formulations with little sandwiching or clustering (Figure 3.17). Similarly, the confusion matrix constructed by joint PCA-LDA reflects poor accuracy across individual 2,4-D formulations (Table 3.8). Accuracy of this model ranged from 15% accuracy in classifying 2,4-D ESTER to 40% accuracy in classifying 2,4-D ACID (Table 3.8). These model accuracies, albeit poor, are largely similar to those produced by analysis of raw spectral data and are markedly less than those reported by Reid (2017).

Practical Implications for Regulatory Management

Increased 2,4-D use will likely lead to a concurrent increase in reports of cotton and soybean injury following OTM of 2,4-D (Bradley 2017; 2018). The potential for injury to be caused by one of many 2,4-D formulations makes managing cases of OTM of 2,4-D difficult. Most of the models in this research demonstrated poor classification performance when challenged with

unknown samples, in contrast to Reid (2017) which reported highly accurate models when analyzing spectra from samples treated with a single 2,4-D concentration at a single evaluation timing. Similarly, improved model accuracies similar to those reported by Reid (2017) were achieved when 2,4-D concentration and evaluation timing were fixed, especially at higher concentrations (data not shown). However, these models are likely not practical or realistic tools for classifying unknown samples due to the sample bias involved in their construction (fixed concentration and evaluation timing.) which would vastly reduce classification efficacy when presented with samples damaged by different concentrations of 2,4-D or harvested at various durations after exposure. The models presented here are based upon spectra of samples exposed to a wide range of 2,4-D concentrations and sampling timings and are likely the best indicators of the real world applicability of this technique towards sample classification. A valid counterpoint may suggest creating several different models that are each fixed over a given concentration and sampling timing and then challenging each model with a spectrum of an unknown sample until identification is achieved, but such an approach would be both costly and inefficient.

This research shows that chemometric analyses of soybean and cotton tissue that have been damaged by various 2,4-D formulations and concentrations and collected at a range of evaluation timings may be useful in identifying 2,4-D formulations in future unknown samples of cotton or soybean tissue, and that utilizing raw or transformed spectral data results in similar classification models. While approaches involving PCA and joint PCA-LDA rarely achieved accuracy over 35% in the present research, utilizing LDA alone to analyze sample spectra can result in classification models capable of identifying 2,4-D formulations in damaged tissue with up to 89% accuracy for specific formulations, and up to 80% accuracy overall. Model accuracy

was often best when identifying 2,4-D CHOLINE or 2,4-D ACID, and worst when identifying 2,4-D DMA or 2,4-D ESTER. Construction of more robust models that cover more concentrations and evaluation timings may be possible in the future, but results suggest joint PCA-LDA modeling may be unsuitable for classifying 2,4-D formulations in damaged crop tissue given that crop exposure may occur with any number of 2,4-D concentrations or sample timings. LDA modeling alone, however, appears to be a viable candidate for future applications. Models based upon a single concentration or evaluation timing, or small ranges thereof may achieve higher accuracy, but in reality have limited applicability given the unknown nature of these variables in unknown samples brought in to the lab for identification. Future research may solve this problem by using HPLC to determine in-plant 2,4-D concentration in ppm before converting this concentration to an approximate herbicide rate (g ae ha^{-1}) based on leaf area of the sample. By estimating approximate herbicide concentration in the sample, the appropriate classification model from the corresponding concentration could be utilized for classification, an approach which has already been shown to be accurate (Reid 2017). This could potentially be the only method currently available for identifying 2,4-D formulation in damaged tissue and an important regulatory tool for managing OTM cases. With further refinement, chemometric analysis of damaged crop tissue may be further developed into an economical and efficient tool for assisting in cases of crop injury following OTM of 2,4-D in the future.

Acknowledgements

No conflicts of interest have been declared. The authors would like to thank members of the Mississippi State Chemistry Laboratory for their assistance and the Mississippi Soybean Promotion Board for funding this research.

Tables

Table 3.1 Location, year, longitude, latitude, elevation, soil type, and planting dates for each experiment.[a]

Location	Year	Longitude	Latitude	Elevation	Soil Type[a]	Planting Date
				m		
Starkville	2017	88°46'W	33°27'N	84	Marietta fine sandy loam	2 May
Starkville	2018	88°46'W	33°27'N	84	Marietta fine sandy loam	1 May

[a]Source: U.S. Department of Agriculture, Natural Resources Conservation Service (2019) http://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspxtable

Table 3.2 Variation explained by each PC following PCA of fingerprint (1800 to 800 cm^{-1}) spectra from cotton or soybean tissue treated 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a]

Data Matrix	Data Type[b]	PC1	PC2	PC3	PC4	PC5
		----------%----------				
Cotton Spectra	Raw	89	7	2	2	-
Cotton Spectra	Transformed	71	12	3	2	2
Soybean Spectra	Raw	69	23	5	1	1
Soybean Spectra	Transformed	83	4	3	2	1

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; PC, principal component; PCA, principal component analysis

[b]Raw spectral data were not normalized, derived or smoothed; Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

Table 3.3 Classification model parameters following LDA alone or joint with PCA of fingerprint spectra (1800 to 800 cm^{-1}) from cotton or soybean tissue treated 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a]

Data Matrix	Data Type[b]	Model Source	Accuracy
			%
Cotton Spectra	Raw	PCA-LDA	33
Cotton Spectra	Raw	LDA	77
Cotton Spectra	Transformed	PCA-LDA	35
Soybean Spectra	Raw	PCA-LDA	36
Soybean Spectra	Raw	LDA	80
Soybean Spectra	Transformed	PCA-LDA	32

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; LDA, linear discriminant analysis; PCA, principal component analysis
[b]Raw spectral data were not normalized, derived or smoothed; Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

Table 3.4 Confusion matrix from the classification model generated by LDA joint with PCA of raw fingerprint spectra (1800 to 800 cm^{-1}) from cotton tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a]

	Actual	ACID	DMA	CHOLINE	ESTER	
Predicted		----------%----------				
ACID		**25**	16	18	9	
DMA		19	**18**	12	10	
CHOLINE		38	33	**44**	36	
ESTER		18	33	26	**45**	
Accuracy (%)		*25*	*18*	*44*	*45*	*33*[†]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; LDA, linear discriminant analysis; PCA, principal component analysis
[†]Weighted average accuracy across all formulation classifications (overall classification model accuracy)

Table 3.5 Confusion matrix from the classification model generated by LDA joint with PCA of transformed fingerprint (1800 to 800 cm^{-1}) from cotton tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a,b]

	Actual	ACID	DMA	CHOLINE	ESTER	
Predicted		----------%----------				
ACID		**31**	19	16	13	
DMA		23	**29**	18	12	
CHOLINE		36	32	**45**	43	
ESTER		10	20	21	**32**	
Accuracy (%)		*31*	*29*	*45*	*32*	*35*[†]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; LDA, linear discriminant analysis; PCA, principal component analysis
[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed
[†]Weighted average accuracy across all formulation classifications (overall classification model accuracy)

Table 3.6 Confusion matrix from the classification model generated by LDA without PCA of raw fingerprint spectra (1800 to 800 cm^{-1}) of cotton tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a]

	Actual	ACID	DMA	CHOLINE	ESTER	
Predicted		----------%----------				
ACID		**73**	13	7	9	
DMA		3	**71**	2	3	
CHOLINE		19	14	**89**	14	
ESTER		5	2	2	**74**	
Accuracy (%)		*73*	*71*	*89*	*74*	*77*[†]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; LDA, linear discriminant analysis; PCA, principal component analysis
[†]Weighted average accuracy across all formulation classifications (overall classification model accuracy)

Table 3.7 Confusion matrix from the classification model generated by LDA joint with PCA of raw fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a]

	Actual	ACID	DMA	CHOLINE	ESTER	
Predicted		----------%----------				
ACID		**45**	25	25	36	
DMA		16	**35**	18	34	
CHOLINE		22	28	**44**	14	
ESTER		17	12	12	**16**	
Accuracy (%)		*45*	*35*	*44*	*16*	*36*[†]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; LDA, linear discriminant analysis; PCA, principal component analysis
[†]Weighted average accuracy across all formulation classifications (overall classification model accuracy)

Table 3.8 Confusion matrix from the classification model generated by LDA joint with PCA of transformed fingerprint (1800 to 800 cm^{-1}) from soybean tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a,b]

	Actual	ACID	DMA	CHOLINE	ESTER	
Predicted		----------%----------				
ACID		**40**	18	30	27	
DMA		26	**36**	28	28	
CHOLINE		16	24	**35**	30	
ESTER		18	22	7	**15**	
Accuracy (%)		*40*	*36*	*35*	*15*	*32*[†]

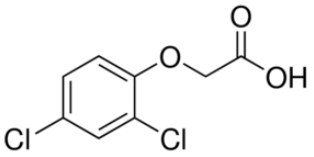
[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; LDA, linear discriminant analysis; PCA, principal component analysis
[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed
[†]Weighted average accuracy across all formulation classifications (overall classification model accuracy)

Table 3.9 Confusion matrix from the classification model generated by LDA without PCA of raw fingerprint spectra (1800 to 800 cm^{-1}) of soybean tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a]

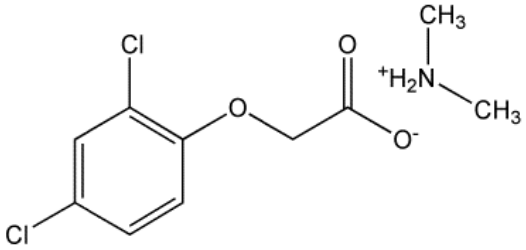
	Actual	ACID	DMA	CHOLINE	ESTER	
Predicted		----------%----------				
ACID		**78**	7	13	14	
DMA		6	**81**	2	14	
CHOLINE		6	7	**80**	6	
ESTER		10	5	5	**66**	
Accuracy (%)		*78*	*81*	*80*	*66*	*80*[†]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; LDA, linear discriminant analysis; PCA, principal component analysis
[†]Weighted average accuracy across all formulation classifications (overall classification model accuracy)

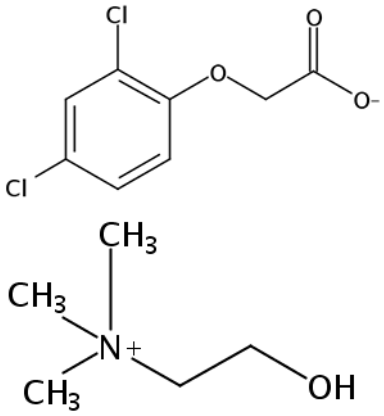
Figures



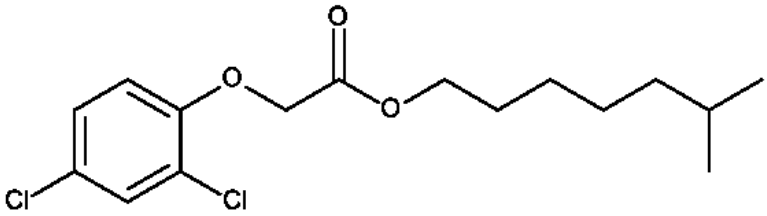
2,4-D acid



Dimethylamine salt of 2,4-D



Choline salt of 2,4-D



2,4-D isooctyl ester

Figure 3.1 Chemical structure of 2,4-D (2,4-dichlorophenoxyacetic acid) alone and formulated with DMA, CHOLINE, or ESTER.[a]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester

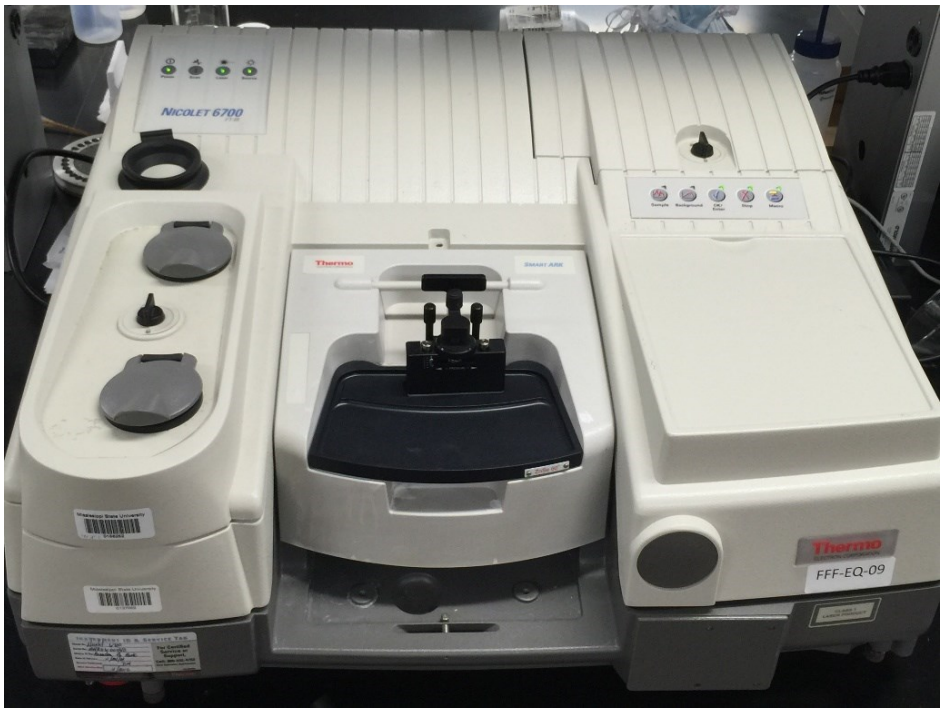


Figure 3.2 Thermo Nicolet 6700 FTIR optical spectrometer equipped with a liquid nitrogen-cooled MCT High-D detector, KBr beamsplitter, and Smart ARK accessory with a ZnSe horizontal attenuated total reflectance crystal at a 60° angle of incidence.

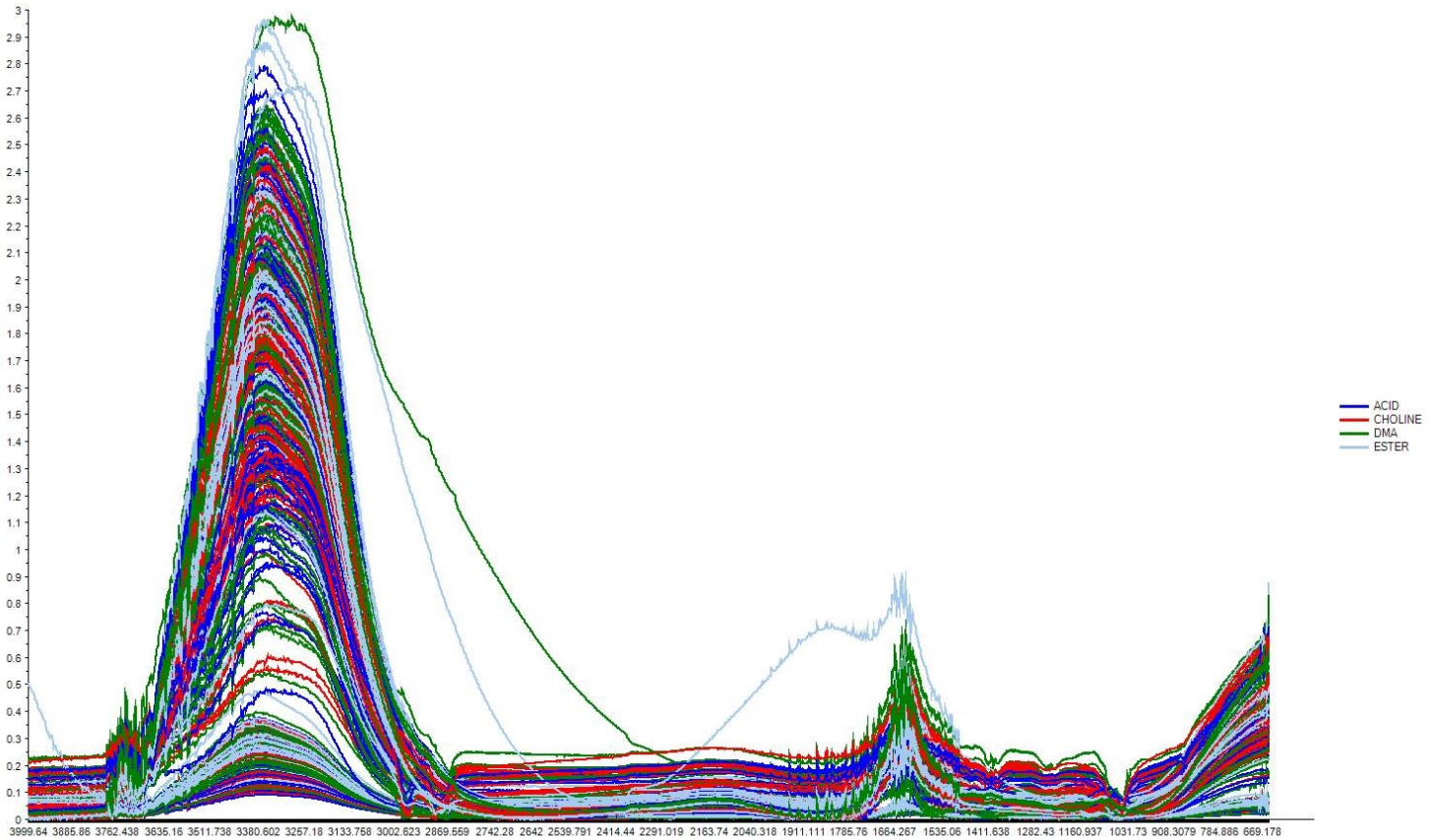


Figure 3.3 Raw spectra (4000 to 650 cm^{-1}) from cotton tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a,b]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester
[b]Y-axis: proportion of infrared light absorbed by the sample (%); X-axis: infrared wavelength (cm^{-1})

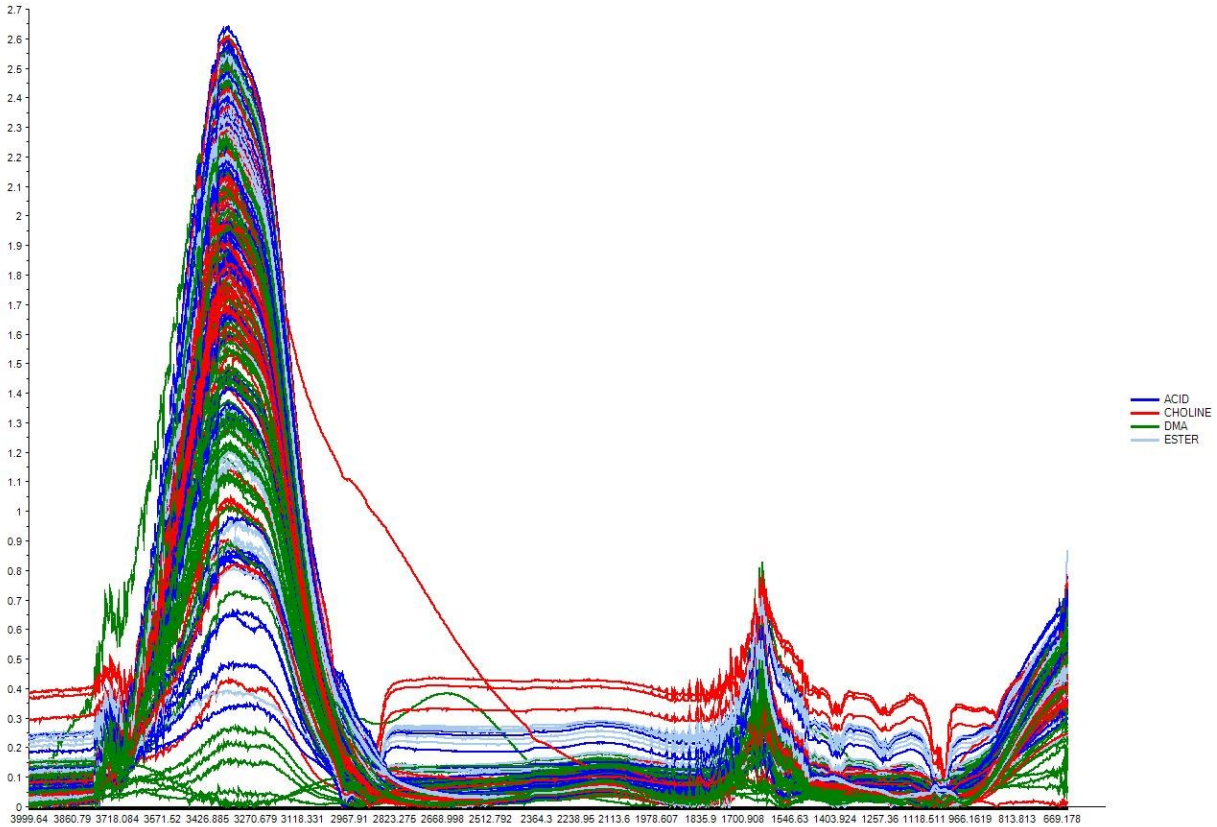


Figure 3.4 Raw spectra (4000 to 650 cm^{-1}) from soybean tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}). [a,b]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester
[b]Y-axis: proportion of infrared light absorbed by the sample (%); X-axis: infrared wavelength (cm^{-1})

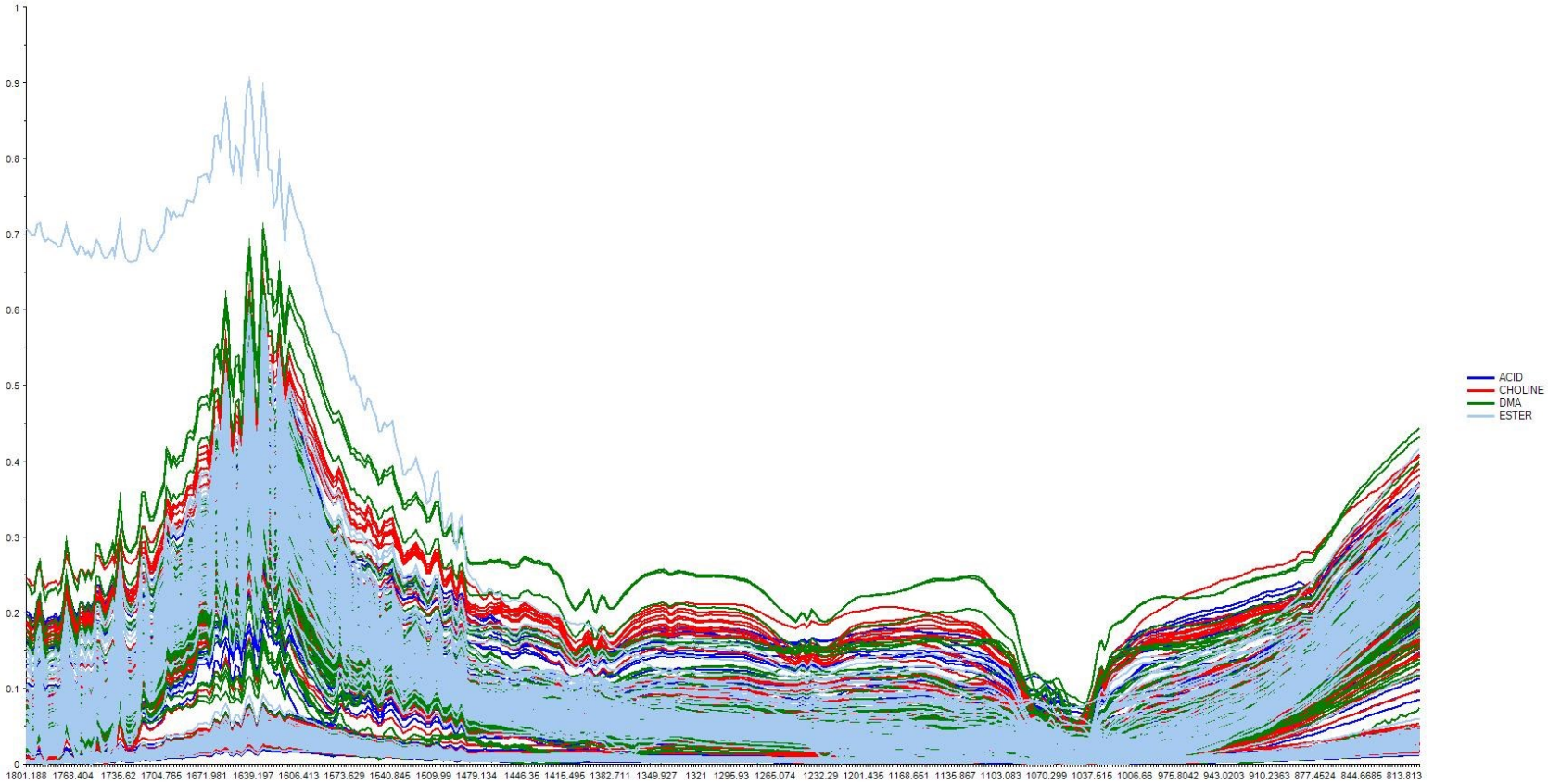


Figure 3.5 Raw fingerprint spectra (1800 to 800 cm^{-1}) from cotton tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}). [a,b]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester
[b]Y-axis: proportion of infrared light absorbed by the sample (%); X-axis: infrared wavelength (cm^{-1})

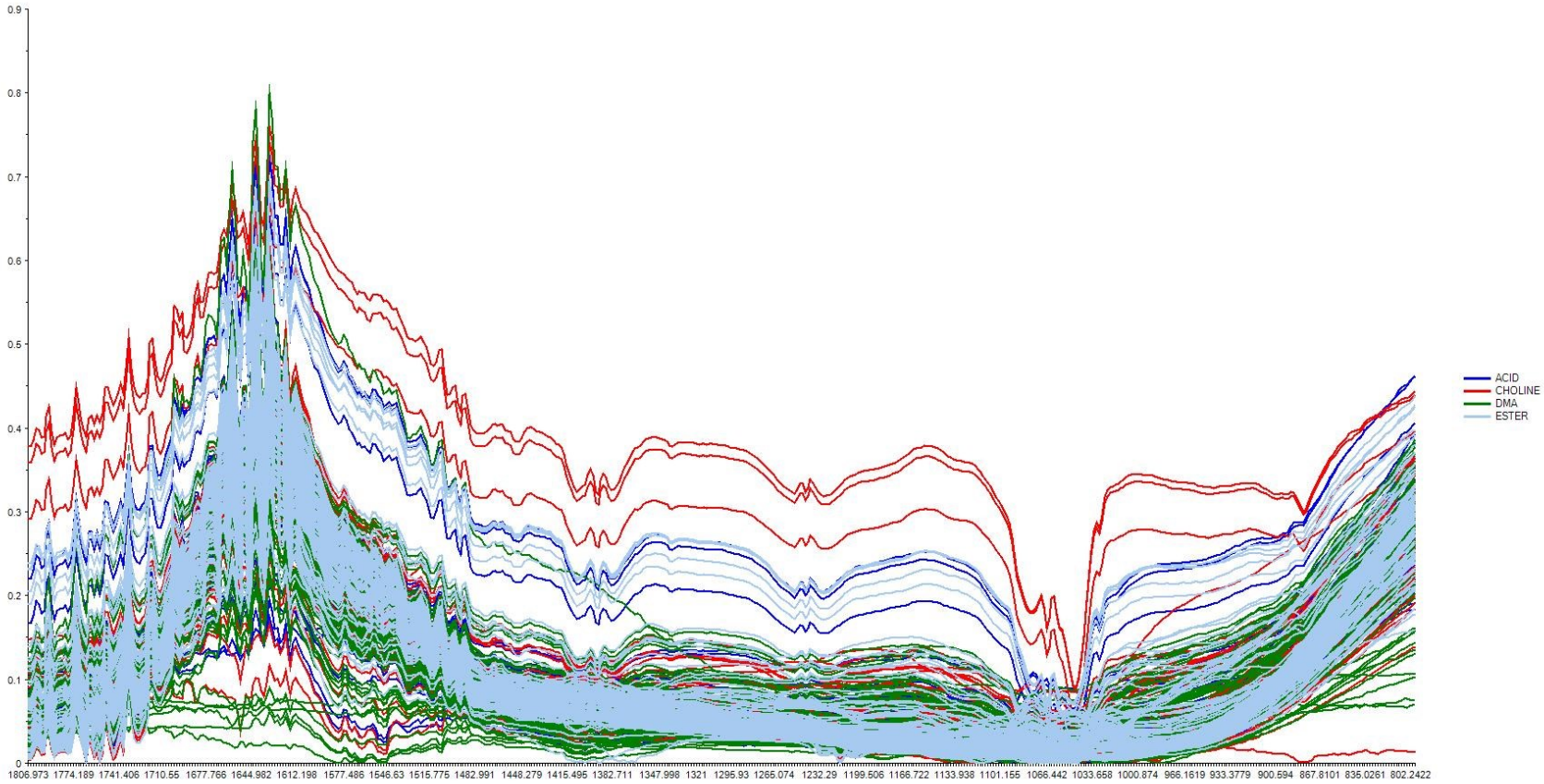


Figure 3.6 Raw fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}). [a,bv]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester
[b]Y-axis: proportion of infrared light absorbed by the sample (%); X-axis: infrared wavelength (cm^{-1})

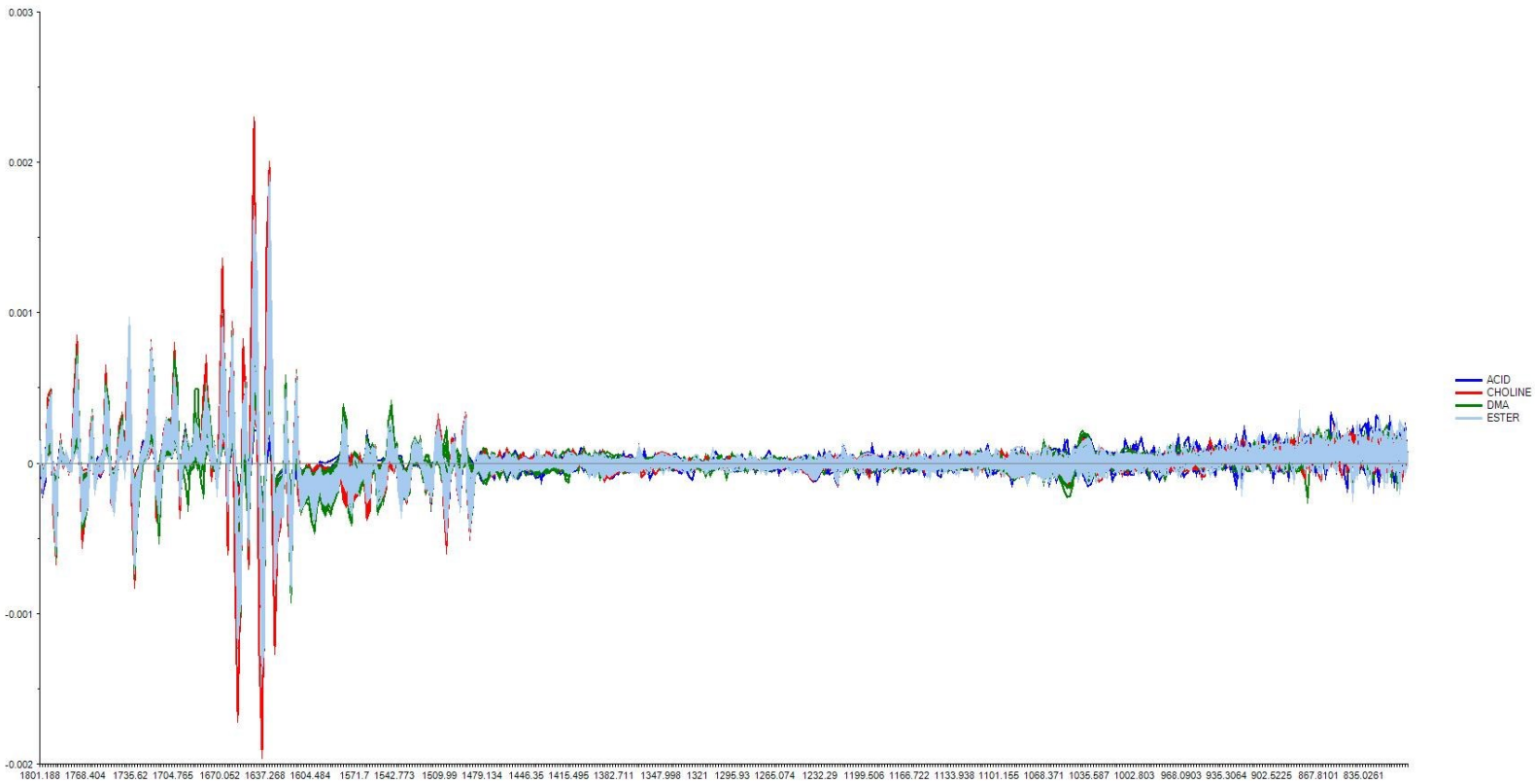


Figure 3.7 Transformed fingerprint spectra (1800 to 800 cm^{-1}) from cotton tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a,b,c]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester
[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed
[c]Y-axis: proportion of infrared light absorbed by the sample (%); X-axis: infrared wavelength (cm^{-1})

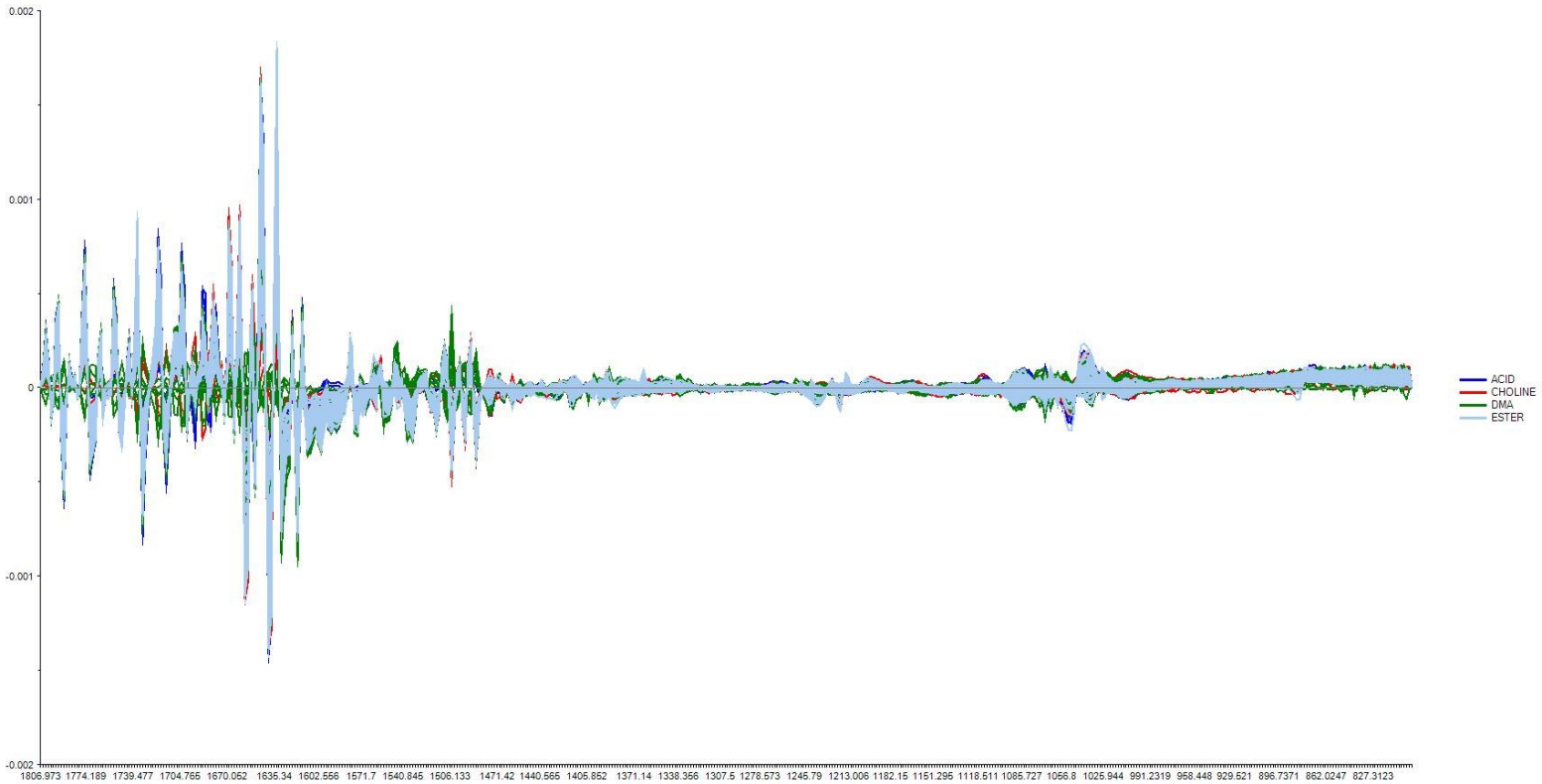


Figure 3.8 Transformed fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a,b,c]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester
[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed
[c]Y-axis: proportion of infrared light absorbed by the sample (%); X-axis: infrared wavelength (cm^{-1})

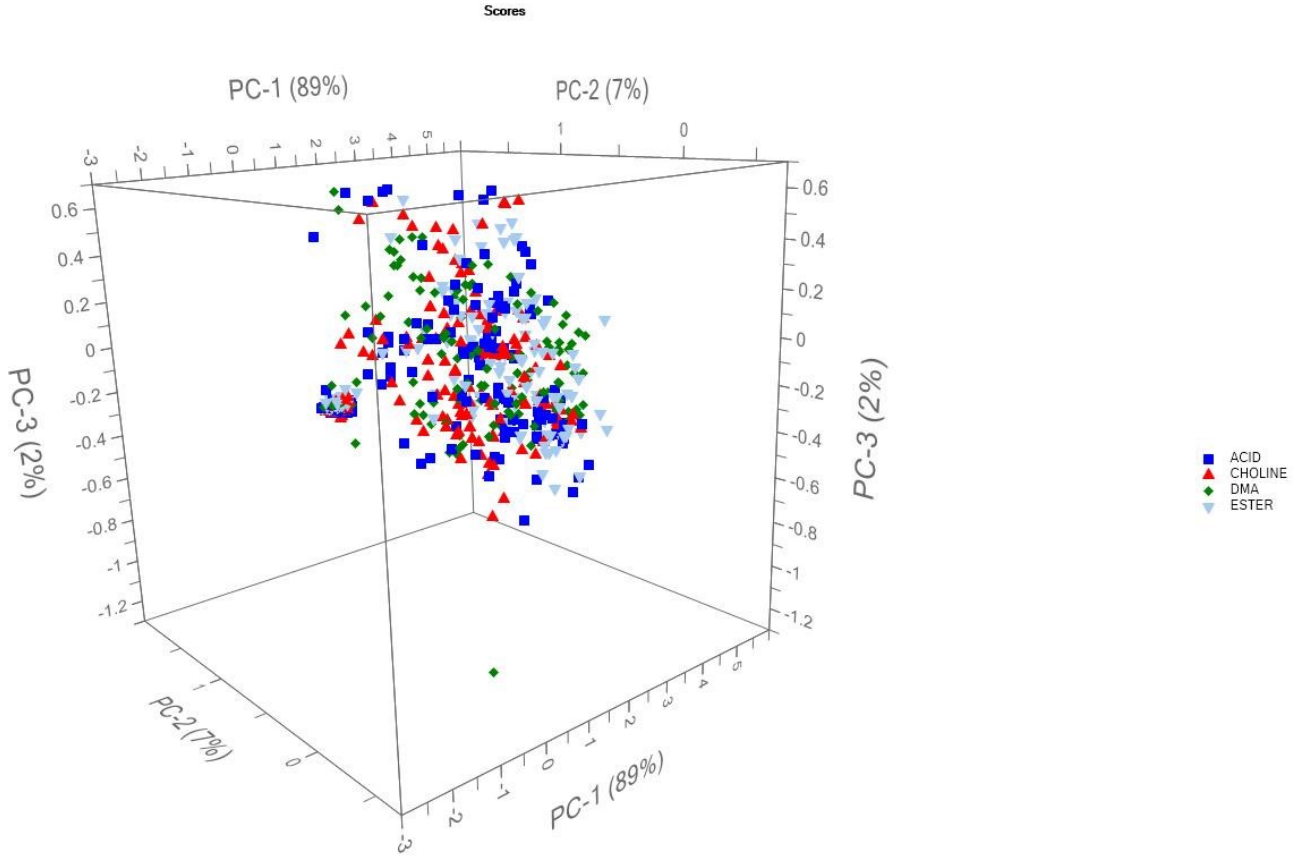


Figure 3.9 3D PCA score plot of raw fingerprint spectra (1800 to 800 cm^{-1}) from cotton tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester

PCA, principal component analysis

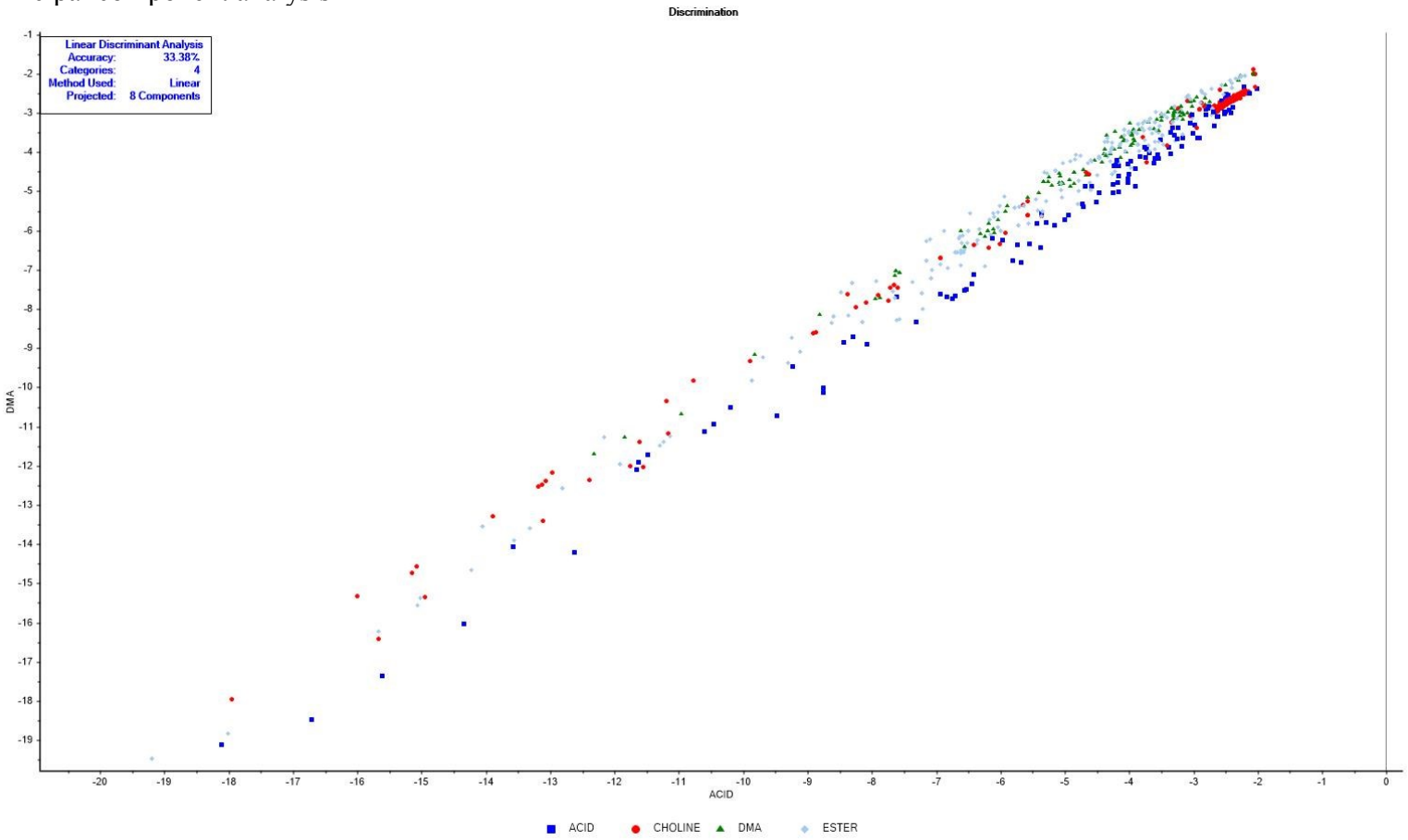


Figure 3.10 LDA discrimination plot following PCA of raw fingerprint spectra (1800 to 800 cm^{-1}) from cotton tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; LDA, linear discriminant analysis; PCA, principal component analysis

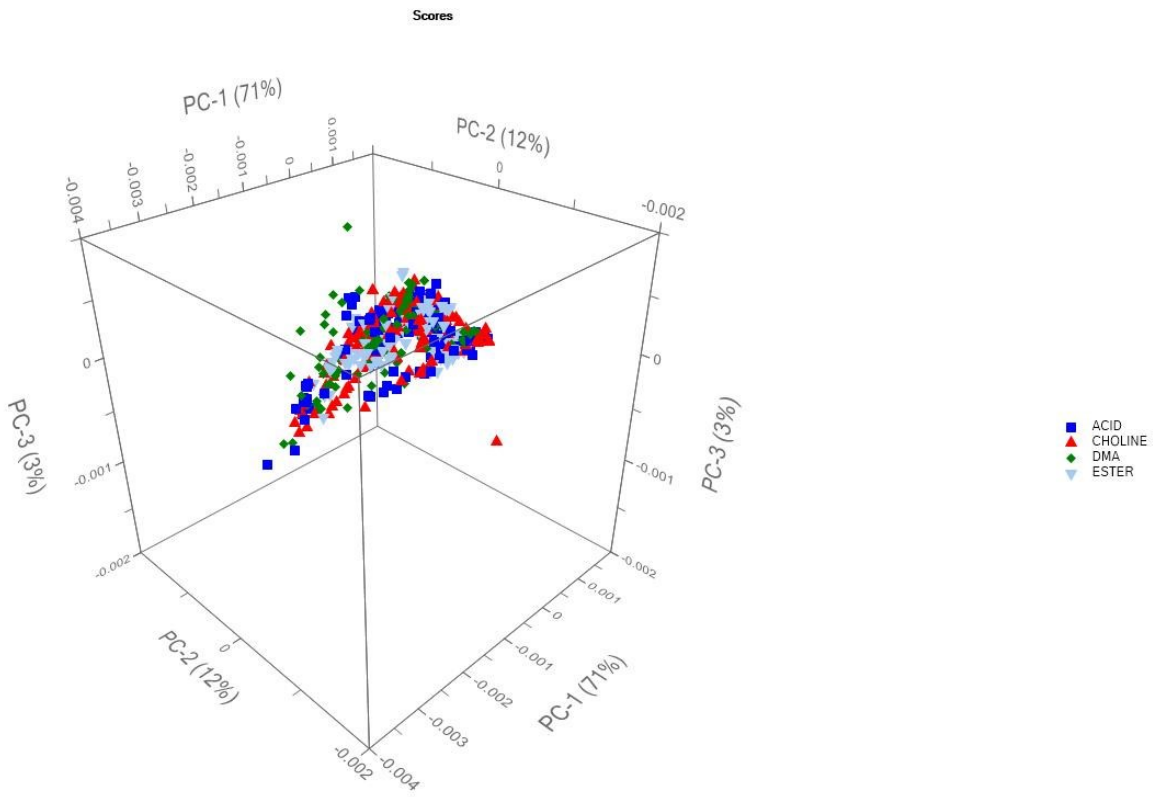


Figure 3.11 3D PCA score plot of transformed fingerprint spectra (1800 to 800 cm^{-1}) from cotton tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a,b]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester
PCA, principal component analysis

[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

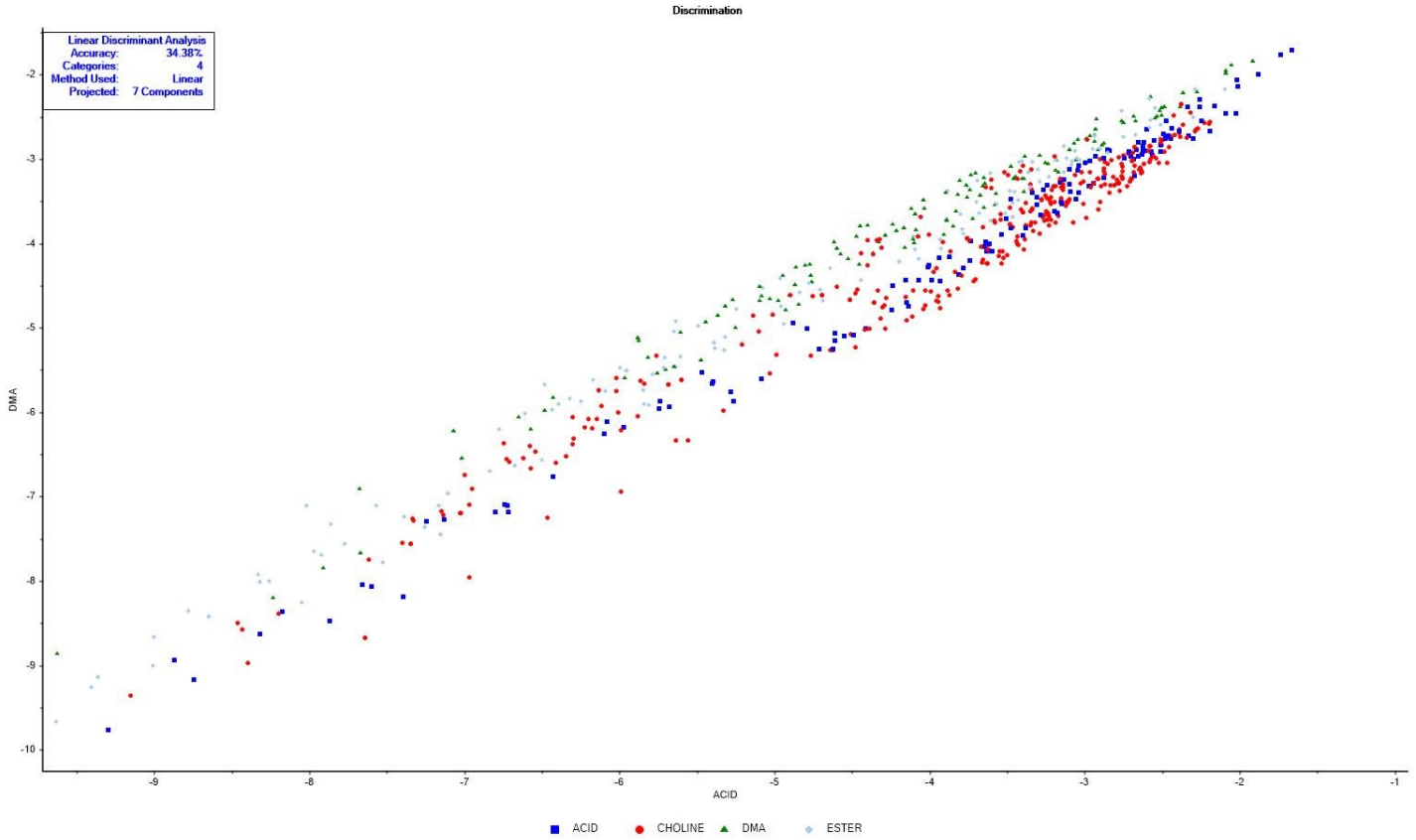


Figure 3.12 LDA discrimination plot following PCA of transformed fingerprint spectra (1800 to 800 cm^{-1}) from cotton tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a,b]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; LDA, linear discriminant analysis; PCA, principal component analysis

[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

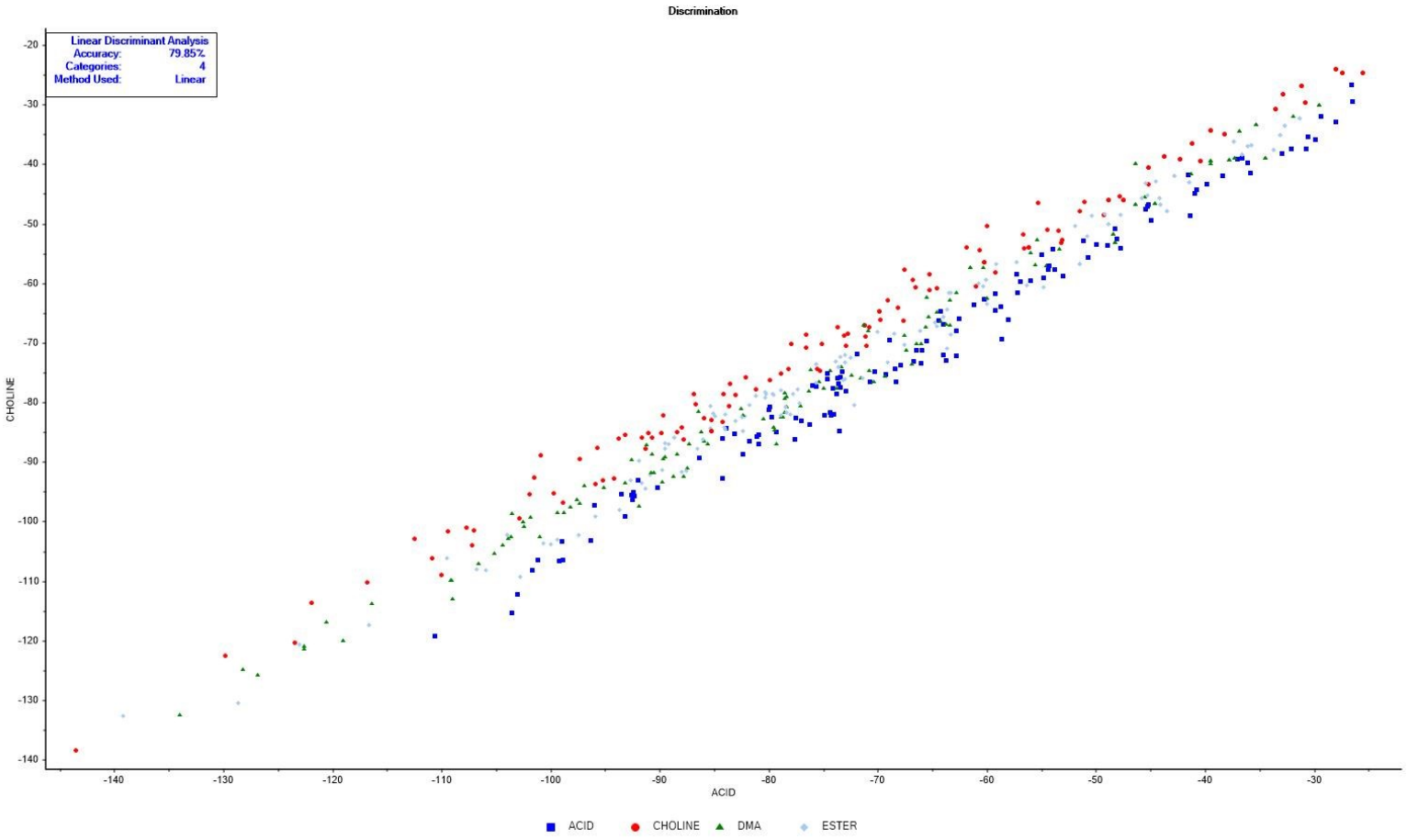


Figure 3.13 LDA discrimination plot without PCA of raw fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; LDA, linear discriminant analysis; PCA, principal component analysis

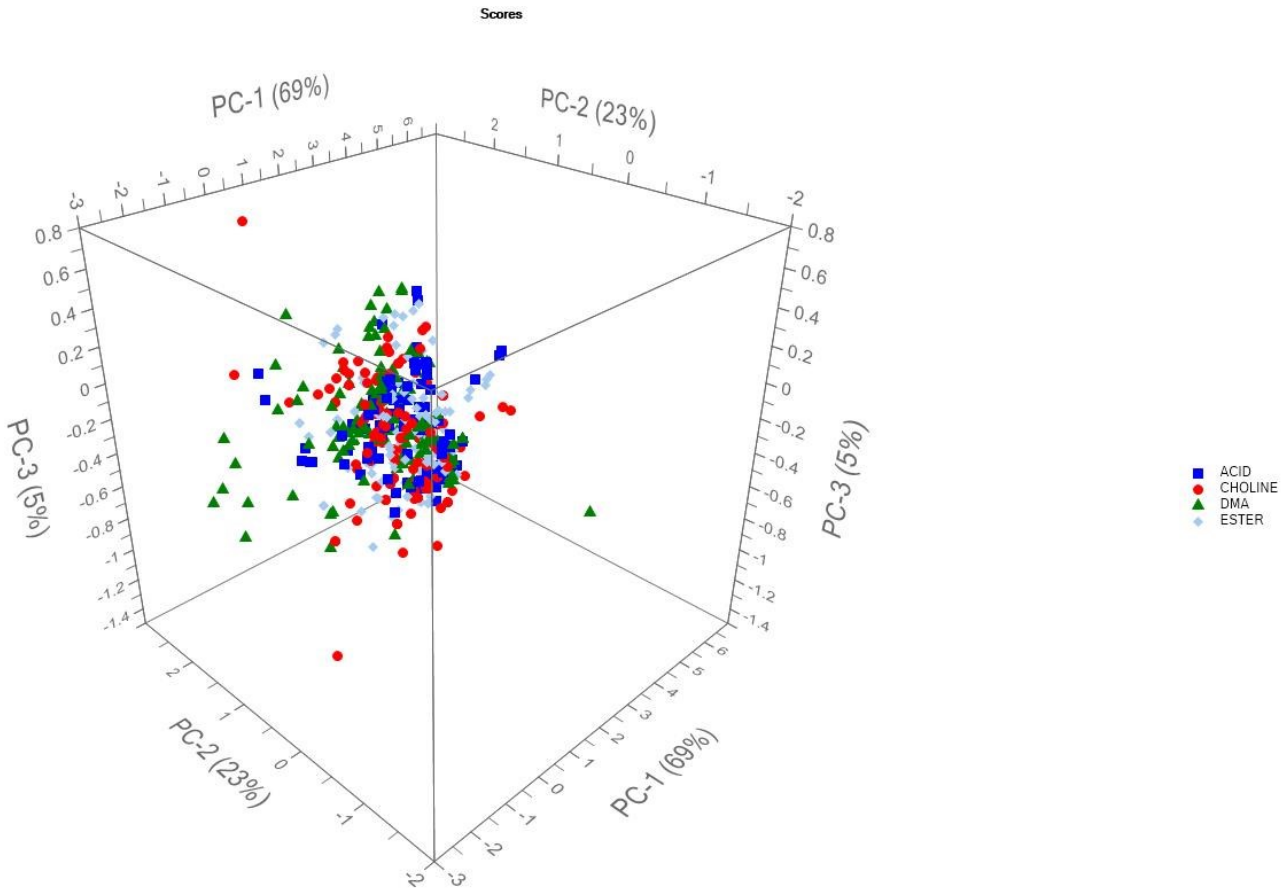


Figure 3.14 3D PCA score plot of raw fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; PCA, principal component analysis

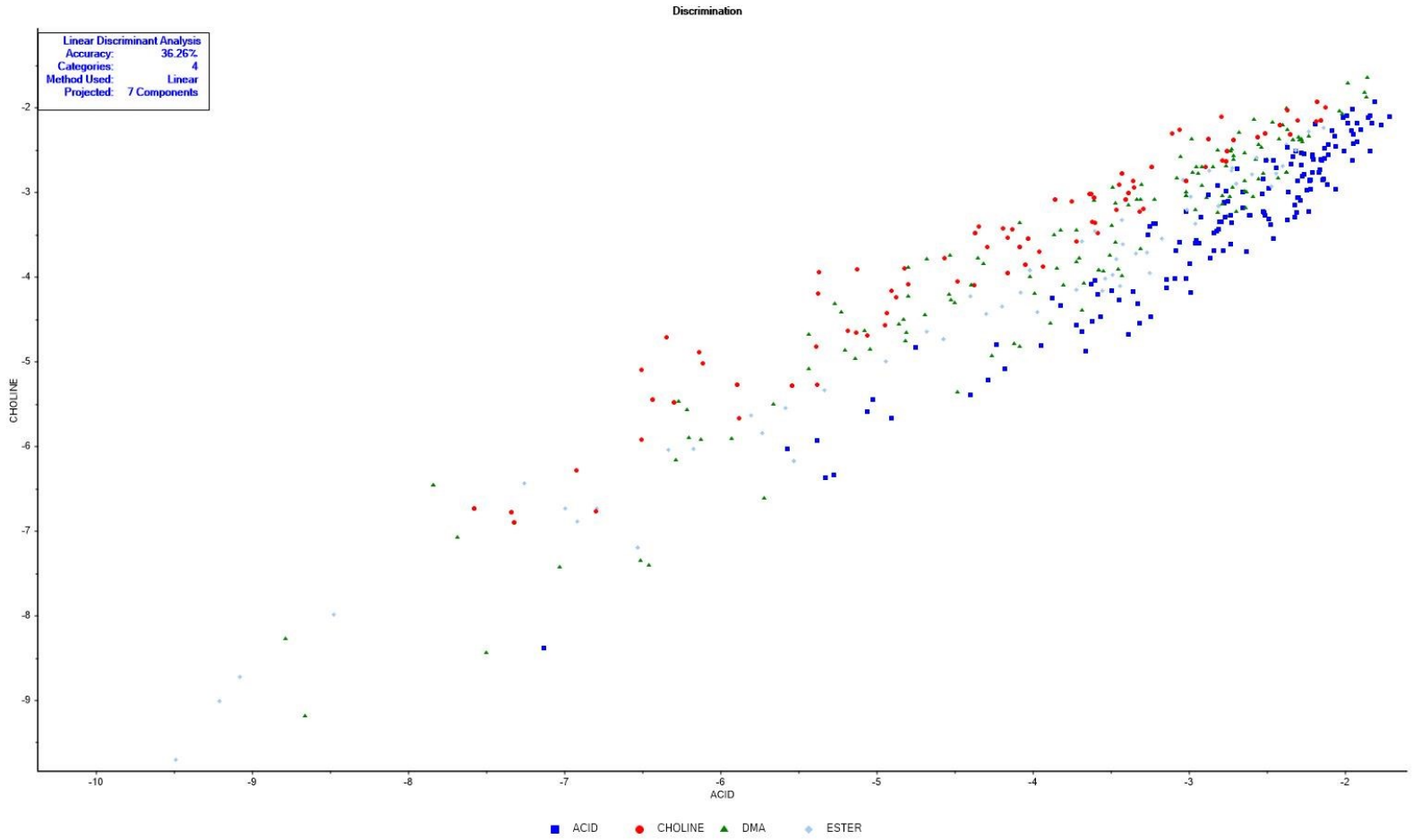


Figure 3.15 LDA discrimination plot following PCA of raw fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; LDA, linear discriminant analysis; PCA, principal component analysis

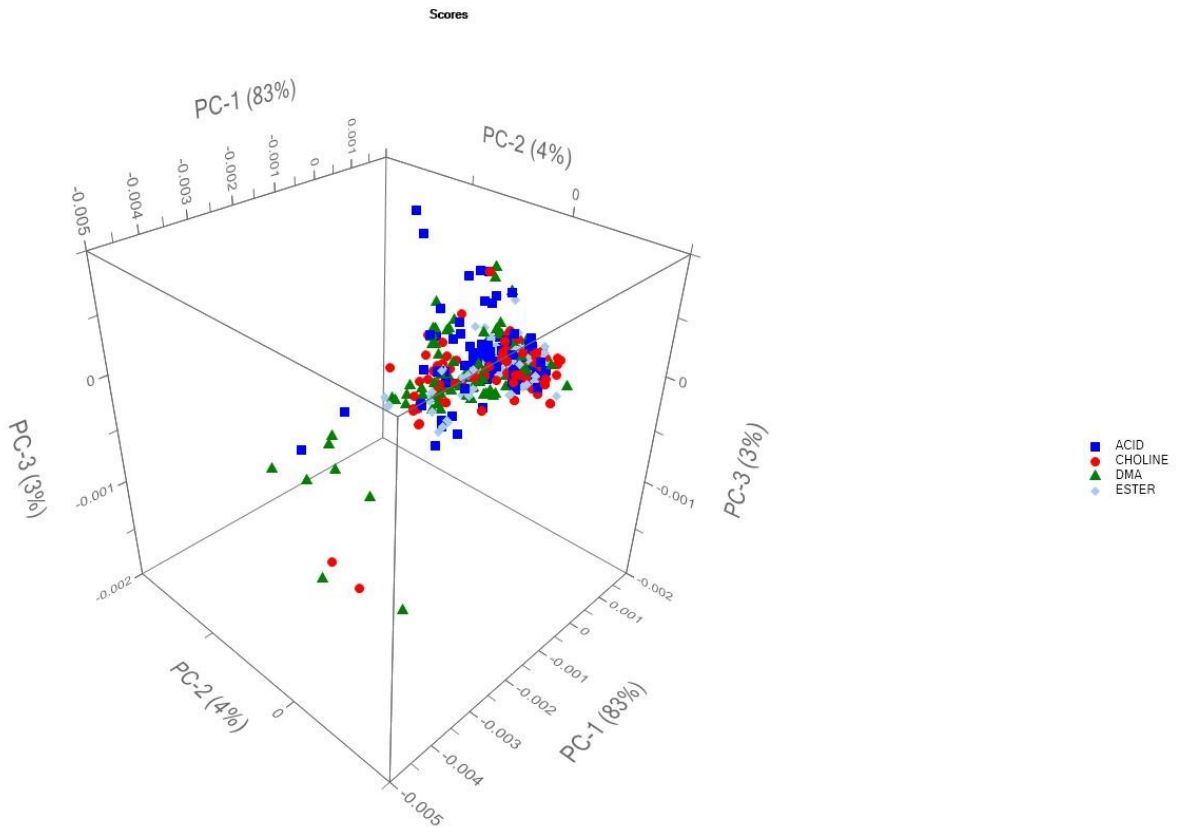


Figure 3.16 3D PCA score plot of transformed fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a,b]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; PCA, principal component analysis
[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

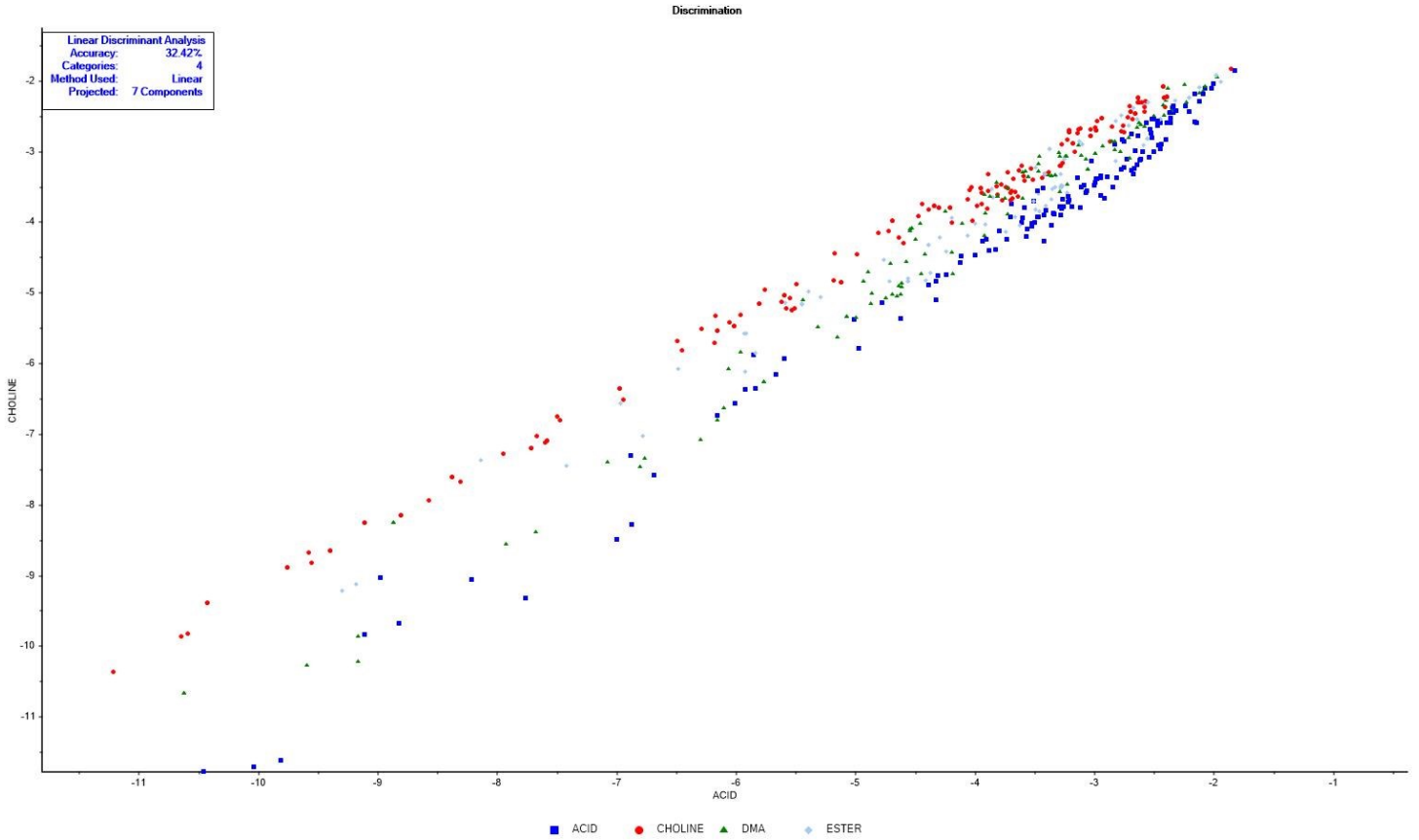


Figure 3.17 LDA discrimination plot following PCA of transformed fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a,b]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; LDA, linear discriminant analysis; PCA, principal component analysis

[b]Transformed spectral data were normalized to the area under the curve, derived to the first Savitzky-Golay derivative, and Savitzky-Golay smoothed

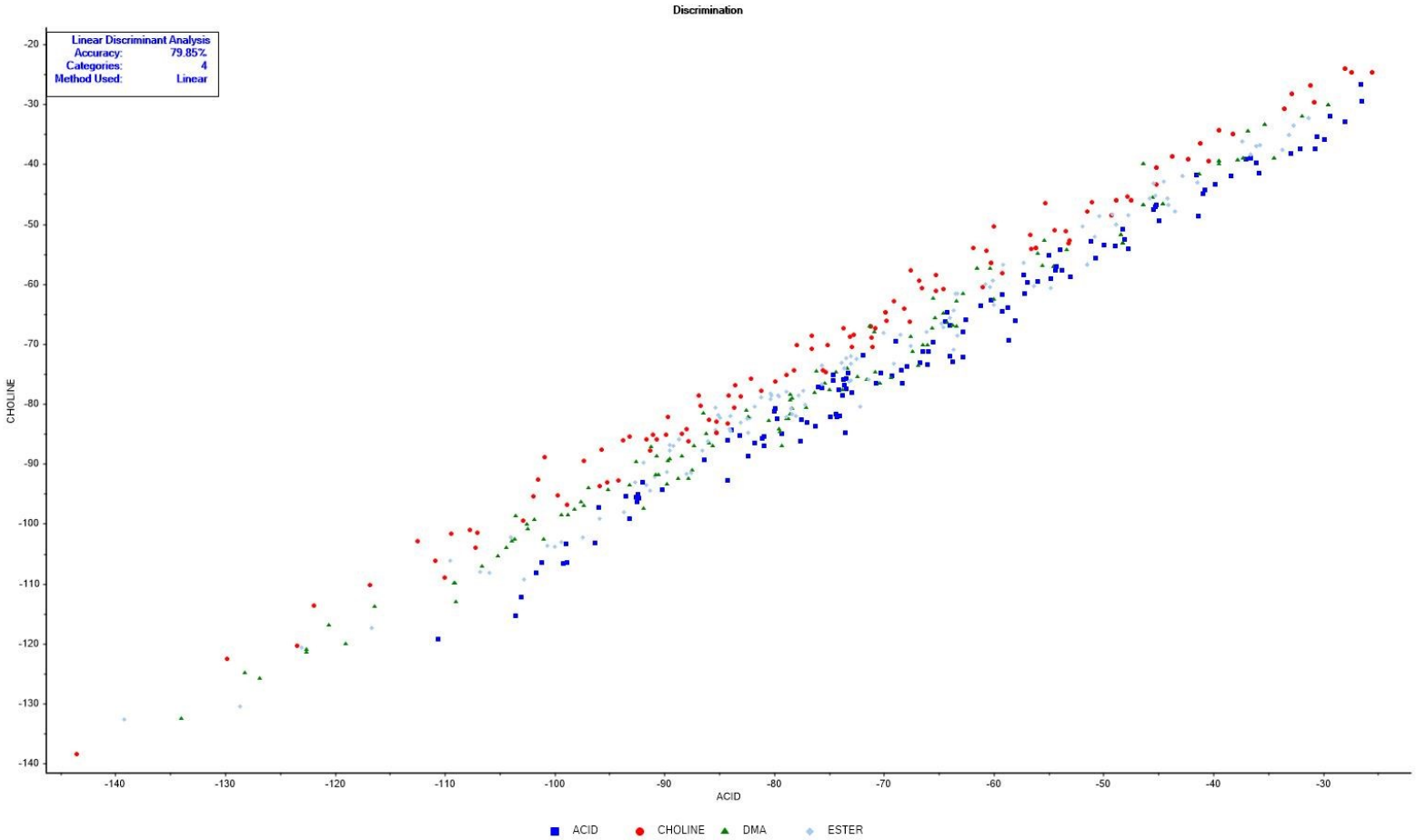


Figure 3.18 LDA discrimination plot without PCA of raw fingerprint spectra (1800 to 800 cm^{-1}) from soybean tissue treated with 2,4-D acid, 2,4-D DMA, 2,4-D CHOLINE, or 2,4-D ESTER pooled over evaluation timing (7, 14, 21, 28, and 56 d after treatment) and 2,4-D concentration (33, 17, 8, 4, 2, 1 g 2,4-D ae ha^{-1}).[a]

[a]Abbreviations: DMA, dimethylamine salt; CHOLINE, choline salt; ESTER, isooctyl ester; LDA, linear discriminant analysis; PCA, principal component analysis

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CHAPTER IV

COTTON AND SOYBEAN RESPONSE TO VARIOUS 2,4-D FORMULATIONS WITH OR WITHOUT GLYPHOSATE

Abstract

Increased 2,4-D use following the commercialization of the Enlist™ Weed Control System may result in more instances of 2,4-D injury from off-target deposition to cotton and soybeans, and these off-target deposition events may involve 2,4-D that has been tank-mixed with glyphosate. Research was conducted in Mississippi to differentiate cotton and soybean response to different formulations of 2,4-D, with or without glyphosate. Older formulations of 2,4-D such as 2,4-D ester or 2,4-D amine generally caused more severe crop response, although the effect of glyphosate presence was more impactful on crop injury. In general, cotton response to 2,4-D exposure was more severe than in soybeans. Yield and height were reduced in both cotton and soybeans if exposure was to 2,4-D tank-mixed with glyphosate as opposed to 2,4-D alone, even though the cultivars used were glyphosate-tolerant. Cotton maturity was delayed by 2,4-D amine relative to 2,4-D choline, and cotton yield was reduced by 2,4-D amine relative to 2,4-D acid. Differences in the magnitude of visible injury due to 2,4-D formulation were not observed in cotton, and were present but narrow enough in soybeans (4%) to be practically undetectable. 2,4-D formulation did affect crop response, but was overshadowed by the effect of glyphosate presence. Differences in crop response due to 2,4-D formulation were narrow enough that differentiating between injury to various formulations will likely remain challenging at the

field level. Development of an analytic approach to differentiating different formulations of 2,4-D causing crop damage may be required in order to properly manage crop injury complaints in the future.

Nomenclature: 2,4-D; cotton, *Gossypium hirsutum* L.; glyphosate; soybean, *Glycine max* (L.) Merr.

Key words: Auxin, deposition, formulation, herbicide interaction, off-target deposition

Introduction

The availability of crop cultivars with resistance to POST applications of 2,4-D has added a necessary tool for controlling herbicide-resistant weed species. The Enlist™ Weed Control System (Corteva Agriscience, Indianapolis, IN 46268) features cotton (*Gossypium hirsutum* L.), corn (*Zea mays* L.), and soybean (*Glycine max* (L.) Merr.) cultivars with engineered resistance to 2,4-D, glyphosate, and glufosinate (Richburg et al. 2012; Wright et al. 2010). While cultivars with tolerance to glyphosate or glufosinate have been available for some time, 2,4-D resistance in these crops is novel and conferred by an aryloxyalkanoate dioxygenase that metabolizes 2,4-D in vivo (Richburg et al. 2012). 2,4-D has been used for effective control of dicotyledonous species for decades in various production systems including cereals, pastures, and turfgrass (Pokorny 1941). However, a sharp increase in 2,4-D use due to adoption of the Enlist™ Weed Control System may occur as producers seek options for controlling increasingly-common herbicide-resistant weeds, especially since there is a relatively low incidence of weed species that exhibit 2,4-D resistance (Heap 2018).

If recent reports of over 1.4 million hectares of soybeans damaged by dicamba in 2017 following the commercialization of dicamba-tolerant crop cultivars (Bradley 2017) are any indicator, off-target deposition (OTD) of 2,4-D onto susceptible crops such as cotton and

soybeans may increase in the near future. Sensitivity of soybeans and especially cotton to 2,4-D has been well-documented for some time (Johnson et al. 2012; Marple et al. 2007, 2008; Sciumbato et al. 2004; Staten 1946). Susceptible cotton and soybeans may be exposed to OTD of 2,4-D via several vectors including herbicide drift, temperature inversions during/after herbicide applications, contaminated spray equipment, and use of older herbicide formulations with high rates of volatility (Boerboom 2004; Cundiff et al. 2017; Egan et al. 2014; Mortensen et al. 2012). While only the choline salt formulation of 2,4-D is labeled for use in the Enlist™ Weed Control System, there are several other 2,4-D formulations available to producers and commonly used in other systems. However, while the formulated 2,4-D active ingredient is different in composition and structure in various products, susceptible crop response is seemingly the same when exposed to a sub-lethal 2,4-D concentration, regardless of formulation. Stem and petiole epinasty, leaf malformation, and necrosis are commonly reported symptoms following exposure to 2,4-D (Johnson et al. 2012; Marple et al. 2007; Sciumbato et al. 2004).

While visible crop injury symptoms following 2,4-D exposure may be similar regardless of 2,4-D formulation, previous research has shown that overall crop response can vary depending on 2,4-D herbicide formulation (Baurle et al. 2015; Sosnoskie et al. 2014; Thompson et al. 2007). In a field volatility experiment, Sosnoskie et al. (2014) reported reduced cotton injury following 48 hr of exposure to 2,4-D choline or 2,4-D amine relative to 2,4-D ester, which also led to greater reductions in cotton ht. Similarly, Bauerle et al. (2015) found that ester formulations of 2,4-D or triclopyr caused more total injury to tomato ((*Solanum lycopersicum* L.) than 2,4-D dimethylamine, 2,4-D acid, triclopyr acid, or various dicamba formulations. Dias et al. (2017) reported increased toxicity of triclopyr trimethylamine in tomato and sunflower (*Helianthus annuus* L.) than triclopyr butoxyethyl ester, triclopyr acid, or triclopyr choline.

Conversely, triclopyr trimethylamine and triclopyr choline resulted in greater soybean injury than the other formulations tested (Dias et al. 2017).

An additional factor influencing crop response to different auxin herbicide formulations is the presence of additional herbicides mixed with the auxin herbicide. Synergistic interactions between herbicides that improve weed control have been reported, such as in Sarabi et al. (2018) which reported increased redroot pigweed (*Amaranthus retroflexus* L.) control following applications of foramsulfuron and nicosulfuron tank-mixed with 2,4-D plus MCPA. Glyphosate is another herbicide commonly tank-mixed with auxin herbicides, and remains a key component of POST weed control in the Enlist™ Weed Control System. Previous research has explored the relationship between glyphosate presence and formulation with auxin herbicide activity. Havens et al. (2018) reported 62% and 91% reductions in drift when 2,4-D choline was pre-mixed with glyphosate dimethylamine instead of 2,4-D dimethylamine mixed with glyphosate potassium and applied with flatfan (TeeJet Extended Range; XR, TeeJet Technologies, Glendale Heights, IL 60139) or air induction (TeeJet Air Induction Extended Range; AIXR, TeeJet Technologies) spray nozzles, respectively. Skelton et al. (2017) observed a 20% reduction in uptake of radiolabeled 2,4-D in corn when 2,4-D acid was applied, as opposed to a premixed formulation of 2,4-D choline plus glyphosate dimethylamine. While the interaction between glyphosate presence and formulation and 2,4-D formulation has been investigated in regards to crop response, further consideration of multiple available 2,4-D formulations is necessary.

Objective

In order to further investigate OTD of 2,4-D and the effect of interaction between different 2,4-D formulations and glyphosate on susceptible species, research was conducted to

assess susceptible cotton and soybean response to four formulations of 2,4-D with or without a glyphosate tank-mix.

Materials and Method

Design and Treatments

Research was conducted from 2015 to 2018 at multiple sites in Mississippi to determine cotton and soybean response to a sub-lethal concentration of four commercially-available 2,4-D formulations with or without a glyphosate tank-mix. Five site-years of research were conducted in cotton, and four in soybeans; site information is shown in Table 4.1. Glyphosate-tolerant, 2,4-D-susceptible cultivars were used. In each site-year, soybean cultivar 'AG 4632' (Bayer Corporation) was seeded at a rate of 328,510 seeds ha^{-1}, and cotton cultivar 'DP 1321 B2RF' (Bayer Corporation) was seeded at a rate of 128,440 seeds ha^{-1} at a 2.5 cm depth. A five by two factorial arrangement of treatments was arranged in a randomized complete block design in each experiment and included four replicates and a non-treated control (NTC). Plots were 12.2 m in length by four 76 cm rows in soybeans or four 97 cm rows in cotton, and were managed according to local recommendations. Experimental factors were 2,4-D product formulation and glyphosate presence. Four 2,4-D formulations were tested: dimethylamine salt (Weedar® 64, Nufarm Americas Inc., Alsip, IL 60803; 'DMA'), 2,4-D acid (Unison®, Helena Agri-Enterprises, LLC, Collierville, TN 38017; 'ACID'), isooctyl ester (Weedone® LV4 EC, Nufarm, 'ESTER'), and choline salt (Enlist One™ Herbicide with Colex D Technology, Corteva Agriscience LLC, Indianapolis, IN 46268; 'CHOLINE'). Glyphosate level was either absent (0 kg ae ha^{-1}) or present (tank mixed at 0.87 kg ae ha^{-1}) as glyphosate potassium salt (Roundup Powermax II™, Bayer Corporation). Glyphosate potassium salt was utilized in this experiment, although Havens et al. (2018) reported reduced OTM of 2,4-D when tank-mixed with bulkier glyphosate salts such

as glyphosate dimethylamine or isopropylamine. Thus, the use of glyphosate potassium salt in this research may be considered a proxy for a potential 'worst case' scenario of OTM of 2,4-D. Herbicide applications were made to V5 soybeans (Fehr and Caviness 1977) and pinhead square cotton (Stewart et al. 2010). 2,4-D was applied at 8.29 g ae ha^{-1} in the cotton experiment (1/64 of the commonly used 0.54 kg ae ha^{-1} rate), a concentration that was identified by a previous study at Mississippi State as sub-lethal but potent enough to cause visible injury on susceptible cotton (Scholtes et al. 2014). This dicamba concentration is also within the range commonly used to simulate off-target 2,4-D deposition (Byrd et al. 2015; Egan et al. 2014; Everitt and Keeling 2009; Johnson et al. 2012). In the soybean experiment, 2,4-D was applied at 132.64 g ae ha^{-1} (1/4 of the common commercial rate). This concentration was also identified by a previous study at Mississippi State as sub-lethal to soybeans but concentrated enough to cause similar injury as the 8.29 g ae ha^{-1} does on cotton (Scholtes et al. 2014). The increased rate of 2,4-D required to damage soybeans is a result of the decreased sensitivity soybeans demonstrate to 2,4-D relative to cotton (Egan et al. 2014; Johnson et al. 2012). Herbicides were applied through a four-nozzle spray boom equipped with TTI11002 spray tips (TeeJet Technologies) 51 cm above the crop canopy operated at 4.8 KPH using a carbon dioxide-pressurized plot backpack sprayer calibrated to deliver 140 L ha^{-1} at 276 kPa. Applications were made to the center two rows of each plot and the outer two rows served as spray buffers. Climate and weather conditions fell within average historical ranges at each site year.

Data Collection and Analyses

Estimates of visible crop injury were made weekly from seven to 28 d after treatment (DAT) on a spectrum of 0 (no visible injury) to 100 (complete plant mortality) (Frans et al. 1986). Nodes above cracked-boll (NACB) counts from the center two rows of each cotton plot

were recorded at boll cut-out, or when the NTC reached five or less nodes above white flower (NAWF) (Bourland et al. 1992; Kerby et al. 1992). Soybean node counts were recorded upon harvest aid application from the center two rows of each plot. Crop ht was recorded from the center two rows of each plot following harvest aid application. Cotton was harvested using a two-row plot picker, and soybean plots were harvested using a small-plot combine.

Cotton and soybean data were analyzed independently. Studentized residuals were utilized to identify values in excess of 2.5, which were removed as outliers prior to ANOVA in order to avoid outlier effects on analysis. All data met appropriate model and distribution assumptions. Crop injury, node counts, ht, and yield data were each subjected to ANOVA using the SAS 9.4 PROC MIXED (SAS Institute, Inc., Cary, NC 27513), and means were separated using Fisher's protected LSD at the $\alpha = 0.05$ level of significance. A NTC (no 2,4-D with no glyphosate) was included in the experiment, but was not included in the analyses in order to minimize MSE and allow greater mean separation among 2,4-D formulations. A separate ANOVA was conducted on data from plots not treated with 2,4-D to determine if glyphosate presence alone affected response. Two-factor interactions between 2,4-D formulation and glyphosate level were analyzed using the SLICE feature of the SAS 9.4 pdmix800 macro to conduct pairwise least squared means comparisons on treatment combination means within PROC MIXED at the $\alpha = 0.05$ significance level (Saxton 1998). 2,4-D formulation and glyphosate concentration were analyzed as fixed effects. Year and location were combined as environment and analyzed as a random effect, as were any effects containing an interaction with environment. All data were thus averaged over environment in order to estimate treatment effects over a wide variety of environments (Blouin et al. 2011; Carmer et al. 1989). This approach

utilizing multiple-environment trials is useful for making broad inferences over time and space (Blouin et al. 2011; Carmer et al. 1989; Walker et al. 2008; Yang 2010).

Results and Discussion

Cotton

The cultivar in this experiment (DeltaPine 1321) expresses resistance to glyphosate (Green 2007). Separate analyses were conducted to investigate any potential effect of glyphosate applied alone (with the blank 2,4-D formulation), and none were detected for any parameter (data not shown). Cotton ht was affected independently by 2,4-D formulation and glyphosate presence ($p = 0.0152, 0.0385,$ respectively). Table 4.2 shows the effect of 2,4-D formulation on cotton ht, NACB, and yield, averaged over glyphosate presence. Table 4.3 displays the effect of glyphosate presence on cotton ht and yield and on soybean visible injury and yield, averaged over 2,4-D formulation. Cotton ht was reduced following exposure to 2,4-D ESTER or 2,4-D DMA and ranged from 99 to 101 cm, relative to 2,4-D ACID (103 cm), and was similar to 2,4-D CHOLINE. Conversely, Sosnoskie et al. (2014) reported reduced ht following exposure to 2,4-D ester relative to 2,4-D CHOLINE, but Bauerle et al. (2015) found 2,4-D ester to be more injurious to tomato than 2,4-D ACID. Exposure to 2,4-D CHOLINE did not affect ht relative to any other 2,4-D formulation (Table 4.2). The addition of glyphosate resulted in a reduction in cotton ht (102 vs 100 cm) when averaged over 2,4-D formulation (Table 4.3).

Visible cotton injury 28 DAT ranged from 44 to 46%, consistent with previous work (Byrd et al. 2015), and was not affected by any experimental factor or interaction thereof ($p = 0.652$). Cotton NACB, which is commonly used as a metric to assess maturity (Bourland et al. 1992; Kerby et al. 1992), was affected by 2,4-D formulation ($p = 0.0252$). It is important to consider that the average NACB count in the NTC in this experiment was nine, implying that

any delay in maturity described here is in addition to the implied delay relative to a NTC. Exposure to 2,4-D DMA resulted in delayed maturity (13 NACB) relative to 2,4-D choline (12 NACB), while exposure to 2,4-D ACID or 2,4-D ESTER did not affect maturity relative to any other formulation (Table 4.2). Exposure to sub-lethal concentrations to 2,4-D leading to delayed maturity is well documented in cotton (Byrd et al. 2015; Marple et al. 2007; McIlrath et al. 1951).

Cotton yield was affected independently by both 2,4-D formulation and glyphosate presence (*p = 0.0001, <0.0001,* respectively). Exposure to 2,4-D ACID or 2,4-D ESTER resulted in increased yield (1,780 to 1,983 kg ha^{-1}) relative to 2,4-D DMA or 2,4-D choline (1,497 to 1,581 kg ha^{-1}), although yield following exposure to 2,4-D ester or 2,4-D choline was simialr (Table 4.2). When averaged over 2,4-D formulation, the addition of glyphosate led to a reduction in seed cotton yield from 1,899 to 1,522 kg ha^{-1} (Table 4.3), a reduction of approximately 20%. However, the yield reduction was much greater relative to the average NTC yield (2850 kg ha^{-1}, data not shown). While the adjuvant load in Roundup Powermax II™ (Bayer Corporation) may be a consideration in causing increased yield loss, the addition of glyphosate alone did not affect cotton yield (*p = 0.6572*). A more likely cause for yield loss due to glyphosate presence in glyphosate-resistant cotton is increased 2,4-D uptake in the presence of glyphosate, which Skelton et al. (2017) demonstrated in field corn.

Soybeans

The addition of glyphosate alone did not affect crop response for any tested parameter (data not shown). Thus, any effect of adding glyphosate is due to the glyphosate augmenting 2,4-D activity and not attributed to the glyphosate itself. Soybean ht and node counts were affected by an interaction between 2,4-D formulation and glyphosate presence (*p = 0.0001, 0.0309*,

respectively). LSD on the multiple comparisons of treatment combination means is displayed in two tables: Table 4.4 shows the effect of glyphosate level within fixed levels of 2,4-D formulation; and Table 4.5 displays the effect of 2,4-D formulation within fixed levels of glyphosate. When each 2,4-D formulation is fixed, the addition of glyphosate reduced soybean ht by an average of seven to eight cm for each formulation except 2,4-D ACID, which was not affected by glyphosate presence (Table 4.4). When glyphosate level is fixed at absent (0 kg ae ha^{-1}), exposure to 2,4-D CHOLINE resulted in taller cotton than 2,4-D ESTER or 2,4-D ACID (86 vs 77 to 80 cm, respectively) (Table 4.5). Exposure to 2,4-D DMA or 2,4-D ESTER resulted in similar cotton ht (80 to 83 cm) (Table 4.5). When glyphosate level is fixed at present (0.87 kg ae ha^{-1}), exposure to 2,4-D ESTER resulted in shorter cotton (72 cm) than exposure to 2,4-D ACID or 2,4-D DMA (76 to 78 cm), and exposure to 2,4-D CHOLINE resulted in similar ht to exposure to 2,4-D DMA or 2,4-D ESTER (Table 4.5). These results somewhat contrast Dias et al. (2017) who found the choline and trimethylamine formulations of triclopyr to be the most injurious to soybeans.

When 2,4-D formulation is fixed, the addition of glyphosate did not affect soybean node count following exposure to any 2,4-D formulation except 2,4-D CHOLINE, in which case the addition of glyphosate led to a reduction in soybean nodes from 16 to 12 (Table 4.4). When glyphosate was absent, exposure to 2,4-D ESTER or 2,4-D ACID reduced soybean node count from 16 to 13 relative to 2,4-D CHOLINE or 2,4-D DMA (Table 4.5). No effects due to 2,4-D formulation were observed when glyphosate was present, and ranged from 12 to 16 (Table 4.5). Soybean fruiting nodes have been shown to be sensitive to 2,4-D exposure (Robinson et al. 2013), and the counts reported here were generally lower than the average NTC node count (18).

Soybean visible injury 28 DAT and yield were affected by glyphosate presence (p = *0.0051, <0.0001*, respectively). When averaged over 2,4-D formulation visible soybean injury 28 DAT was increased by four percent if glyphosate was present, and the range of visible injury (22 to 26%) was consistent with Robinson et al. (2013), which characterized soybean response to several concentrations of 2,4-D (Table 4.3). Similarly, when averaged over 2,4-D formulation, soybean yield was reduced from 3,199 kg ha^{-1} to 2,627 kg ha^{-1} when glyphosate was present (Table 4.3). This yield reduction was greater compared to the average NTC yield (3340 kg ha^{-1}).

Conclusions

Exposure to a sub-lethal concentration of 2,4-D will cause deleterious effects in susceptible soybean and especially cotton cultivars. The magnitude of crop response will vary depending on the 2,4-D formulation involved and whether or not glyphosate is present, even if the cultivars are resistant to glyphosate. However, the degree of differentiation between cotton or soybean response to different 2,4-D formulations may be too narrow to discern at the field level. In general, exposure to older formulations of 2,4-D such as 2,4-D ester or 2,4-D dimethylamine appears to be more injurious to soybeans and cotton than other formulations, although the effect of different formulations is markedly less impactful on crop response than glyphosate presence.

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Tables

Table 4.1 Location, year, longitude, latitude, elevation, soil type, and planting and harvest dates for each experiment site-year.

Location	Year	Longitude	Latitude	Elevation	Soil Type[a]	Planting Date		Harvest Date	
				m		cotton	soybeans	cotton	soybeans
Brooksville	2015	88°34'W	33°15'N	85	Brooksville silty clay	21 May	-	21 October	-
Brooksville	2016	88°32'W	33°15'N	76	Okolona silty clay	26 April	-	7 October	-
Brooksville	2017	88°32'W	33°15'N	76	Okolona silty clay	19 May	-	24 October	-
Starkville	2015	88°46'W	33°28'N	81	Catalpa silty clay loam	4 May	-	9 September	-
Starkville	2016	88°46'W	33°28'N	81	Catalpa silty clay loam	26 April	-	27 September	-
Starkville	2017	88°46'W	33°27'N	84	Marietta fine sandy loam	-	2 May		2 October

Table 4.1 (continued)

Starkville	2018	88°46'W	33°27'N	84	Marietta fine sandy loam	-	1 May	-	25 September
West Starkville	2018	88°86'W	33°49'N	83	Leeper silty clay loam	-	10 May	-	25 September
Scott	2018	91°07'W	33°59'N	39	Robinsonville-Crevasse silty clay loam	-	23 May	-	4 October

[a]Source: U.S. Department of Agriculture, Natural Resources Conservation Service (2018) http://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx

Table 4.2 The effect of formulation of 8.29 g 2,4-D ae ha^{-1} on cotton height, NACB, and yield averaged over glyphosate presence (0 or 0.87 kg ae ha^{-1}) when exposed at the pinhead square growth stage.[a,b,c]

Formulation	Height	NACB	Yield
	(cm)	(count)	(kg ha^{-1})
ACID	103 a	13 ab	1,983 a
CHOLINE	101 ab	12 b	1,581 bc
ESTER	101 b	13 ab	1,780 ab
DMA	99 b	13 a	1,497 c

[a] Abbreviations: ACID, 2,4-D acid; CHOLINE, 2,4-D choline; ESTER, 2,4-D isooctyl ester; DMA, 2,4-D dimethylamine; NACB, nodes above cracked boll

[b] Values within a column that share a letter are not different based on Fisher's protected LSD at $p \leq 0.05$

[c] Values are generated as estimates by PROC MIXED and then rounded to the nearest whole number, resulting in some cases where two of the same values may have different mean separation groupings due to whether they have been rounded up or down to the presented value

Table 4.3 The effect of tank-mixing 0.87 kg glyphosate ae ha^{-1} with 8.29 g 2,4-D ae ha^{-1} on cotton ht and yield and on soybean injury and yield following exposure to pinhead square cotton or V5/V6 soybeans averaged over 2,4-D formulation.[a,b]

	Cotton		Soybeans	
Glyphosate Concentration	Height	Yield	Injury	Yield
(kg ae ha^{-1})	(cm)	(kg ha^{-1})	(%)	(kg ha^{-1})
0.00	102 a	1,899 a	22 b	3,199 a
0.87	100 b	1,522 b	26 a	2,627 b

[a]Values within a column that share a letter are not different based on Fisher's protected LSD at $p \leq 0.05$

[b]Cotton yield is presented as machine-harvested seed cotton wt

Table 4.4 The effect of tank-mixing 0.87 kg glyphosate ae ha^{-1} with 8.29 g ae 2,4-D ha^{-1} formulated as acid, choline salt, isooctyl ester, or dimethylamine on soybean height and node counts following exposure at the V5/V6 growth stage.[a,b,c]

2,4-D Formulation	Glyphosate Concentration	Height	Node
	(kg ae ha^{-1})	(cm)	(count)
ACID	0	77 a	13 a
	0.87	78 a	14 a
CHOLINE	0	86 a	16 a
	0.87	74 b	12 b
ESTER	0	80 a	13 a
	0.87	73 b	16 a
DMA	0	83 a	16 a
	0.87	76 b	14 a

[a] Abbreviations: ACID, 2,4-D acid; CHOLINE, 2,4-D choline; ESTER, 2,4-D isooctyl ester; DMA, 2,4-D dimethylamine

[b] Values within a column that share a letter are not different based on Fisher's protected LSD at $p \leq 0.05$

[c] Values within a column are to be compared within the same dicamba formulation, denoted by each subsection bounded by dashed lines

Table 4.5 The effect of formulation of 8.29 g 2,4-D ae ha^{-1} with or without 0.87 kg ae glyphosate ha^{-1} on soybean height, visible injury, and node counts following exposure at the V5/V6 growth stage. [a,b]

Glyphosate Concentration	2,4-D Formulation	Height	Nodes[c]
(kg ae ha^{-1})		(cm)	(count)
0	ACID	77 c	13 b
	CHOLINE	86 a	16 a
	ESTER	80 bc	13 b
	DMA	83 ab	16 a
0.87	ACID	78 a	14 a
	CHOLINE	74 ab	12 a
	ESTER	72 b	16 a
	DMA	76 a	14 a

[a] Abbreviations: ACID, 2,4-D acid; CHOLINE, 2,4-D choline; ESTER, 2,4-D isooctyl ester; DMA, 2,4-D dimethylamine

[b] Values within a column that share a letter are not different based on Fisher's protected LSD at $p \leq 0.05$

[c] Node counts are generated as estimates by PROC MIXED and then rounded to the nearest whole number, resulting in some cases where two of the same values may have different mean separation groupings due to whether they have been rounded up or down to the presented value

[d] Values within a column are to be compared within the same glyphosate concentration, denoted by each subsection bounded by dashed line

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CHAPTER V

FIELD EVALUATION OF COTTON AND SOYBEAN RESPONSE TO VARIOUS DICAMBA FORMULATIONS WITH OR WITHOUT GLYPHOSATE

Abstract

Increased dicamba use has led to numerous incidences of off-target movement onto susceptible crops. Multiple dicamba formulations are available and are often tank-mixed with glyphosate. Susceptible species may respond differently to different dicamba formulations with or without glyphosate. Research was conducted to characterize the response of glyphosate-tolerant, dicamba-susceptible cotton and soybeans to a sub-lethal concentration of various dicamba formulations with and without glyphosate. In cotton, glyphosate presence led to a 20% yield reduction following dicamba exposure, and ht was reduced by dicamba diglycolamine (DGA), N,N-Bis-(3-aminopropyl) methylamine (BAPMA), and dimethylamine (DMA) relative to DGA with potassium acetate (DGAAC). Soybeans were more sensitive than cotton to all dicamba formulations. Glyphosate presence and dicamba formulation affected soybean yield, which was reduced most by dicamba DMA or by tank-mixing glyphosate with dicamba. Minimal effects were observed on soybean node counts. When formulation was fixed, inclusion of glyphosate led to increased injury with all formulations except dicamba DGAAC or BAPMA, which were unaffected by glyphosate presence. When glyphosate was absent, no differences in soybean injury occurred due to dicamba formulation, however, dicamba

DGA and DMA led to increased injury when glyphosate was present. Soybean ht was generally reduced by inclusion of glyphosate when formulation was fixed, and greater ht effects due to dicamba formulation at fixed glyphosate levels were ambiguous. In general, the presence of glyphosate and use of the DMA dicamba formulation exacerbates crop response relative to the newer formulations dicamba DGAAC or dicamba BAPMA, though differences may be subtle and difficult to observe at the field level.

Nomenclature: Cotton, *Gossypium hirsutum* L. ; dicamba; glyphosate; soybean, *Glycine max* (L.) Merr.

Key words: Auxin, deposition, formulation, herbicide interaction, off-target movement, tank contamination

Introduction

The development of 255 herbicide-resistant weed species, including 43 with resistance to glyphosate and 160 with resistance to ALS inhibitors (Heap 2018) has contributed to private development of new weed control technologies. The commercialization of the Roundup Ready® Xtend Crop System (Bayer Corporation, 100 Bayer Boulevard, Whippany, NJ 07981) allows for the use of POST applications of dicamba for weed control in cotton (*Gossypium hirsutum* L.), soybeans (*Glycine max* (L.) Merr.), and corn (*Zea mays* L.). Five herbicides with the same dicamba active ingredient formulated differently are available for use in the Roundup Ready® Xtend Crop System, and include Xtendimax® herbicide with VaporGrip® Technology and Roundup Xtend™ herbicide with VaporGrip® Technology [pending regulatory approvals] (Bayer Corporation), Engenia™ (BASF Corporation, 100 Park Ave., Florham Park, NJ 07932), and

FeXapan™ (DuPont USA, 1007 Market Street, Wilmington, DE 19898). While only these five herbicides are available for legal use in the Xtend Crop System, many other dicamba-containing herbicides are available to producers for use in other systems such as range and pasture weed control, burndown weed control, or rights-of-way. Susceptible soybean and cotton cultivars demonstrate similar symptomology regardless of the specific product formulation involved in the exposure event: leaf cupping and strapping, stem and petiole epinasty, and callus formation on stems (Egan et al. 2014; Marple et al. 2007; Sciumbato et al. 2004). Scenarios involving the off-target movement (OTM) or off-target deposition (OTD) of sub-lethal concentrations of auxin herbicides can arise in many ways, primarily herbicide drift, volatility, spray-tank contamination, or temperature inversion events (Boerboom 2004; Cundiff et al. 2017; Egan et al. 2014; Mortensen et al. 2012). The increased use of dicamba following the commercialization of the Roundup Ready® Xtend Crop System has resulted in a multitude of dicamba OTM events causing widespread damage in the U.S. As of October 15, 2017, approximately 1.5 million ha of U.S. soybeans have been damaged by dicamba (Bradley 2017). As of July 15, 2018, an estimated 445,000 ha of soybeans in the U.S. were injured by dicamba (Bradley 2018).

Differential effects on susceptible crops of various formulations of auxin herbicides have been reported previously and to varying degrees. Thompson et al. (2007) found that soybean exposure to 2,4-D ester led to greater injury (10%) in one site-year than 2,4-D amine (3%), although injury from either was less than injury following exposure to dicamba diglycolamine. Furthermore, differences in plant-back restrictions following early preplant applications on various dicamba herbicide labels such as dicamba dimethylamine (Rifle®, Loveland Products, Loveland, CO 80538) and dicamba

diglycolamine (21 d vs 14 d, respectively) may indicate differential soil activity between various formulations of the same parent auxin (Thompson et al. 2007). Mueller et al. (2013) demonstrated that the amount of dicamba dimethylamine present in the air 48 hr after application was twice the value of dicamba diglycolamine when evaluated under field conditions. Similarly, Egan and Mortensen (2012) reported that dicamba dimethylamine vapor could be detected at 0.56 g ae ha^{-1} 21 m from the treated plot, and that in contrast vapor drift was reduced by 94% by dicamba diglycolamine. Sosnoskie et al. (2014) reported increased cotton injury and reduced height following 48 hr of exposure to 2,4-D ester relative to 2,4-D amine or 2,4-D choline in a field experiment set up to simulate 2,4-D volatility. Bauerle et al. (2015) reported increased tomato (*Solanum lycopersicum* L.) injury following exposure to ester formulations of 2,4-D or triclopyr relative to 2,4-D dimethylamine, 2,4-D acid, dicamba dimethylamine, dicamba acid, dicamba diglycolamine, and triclopyr acid. Dias et al. (2017) tested four formulations of the auxin herbicide triclopyr and found that the trimethylamine salt formulation provided a lower ED_{50} in tomato and sunflower (*Helianthus annuus* L.) than butoxyethyl ester, pyridinyloxyacetic acid or choline salt formulations. Additionally, the trimethylamine and choline salt triclopyr formulations induced greater injury on soybeans, whereas no differences in cotton response due to triclopyr formulation were reported (Dias et al. 2017).

Tank-mixing herbicides can lead to synergistic weed control, such as with foramsulfuron and nicosulfuron tank-mixed with 2,4-D plus MCPA providing increased control of redroot pigweed (*Amaranthus retroflexus* L.) (Sarabi et al. 2018). Similarly, the presence and formulation of glyphosate tank- or pre-mixed with auxin herbicides has

been shown to affect off-target movement and deposition of auxin herbicides (Havens et al. 2018; Skelton et al. 2017). Glyphosate remains a staple of POST weed control programs, and is labeled for use in the Xtend Crop System, which contains cultivars with engineered tolerance to glyphosate (and glufosinate in cotton) in addition to dicamba tolerance (Feng et al. 2014; Wright et al. 2010). Application of glyphosate dimethylamine pre-mixed with 2,4-D choline led to a reduction in drift by 62% and 91% relative to a tank-mix of 2,4-D dimethylamine plus glyphosate potassium when applied with flatfan (TeeJet Extended Range; XR, TeeJet Technologies, Glendale Heights, IL 60139) or air induction (TeeJet Air Induction Extended Range; AIXR, TeeJet Technologies) spray nozzles, respectively, and provided similar drift reduction to an air induction nozzle (TeeJet TurboTeeJet Induction; TTI, TeeJet Technologies) (Havens et al. 2018). Skelton et al. (2017) reported a 20% increase of radiolabeled 2,4-D uptake in corn (*Zea mays* L.) when a premixed formulation of 2,4-D choline plus glyphosate dimethylamine was used compared to 2,4-D acid, although glyphosate did not affect 2,4-D metabolism. Kelley et al. (2005) reported synergistic interaction between dicamba diglycolamine and glyphosate isopropylamine causing increased soybean injury, decreased soybean height, and decreased yield relative to dicamba alone in one site-year of research, and in contrast to imazethapyr, imazamox, and fomesafen, which each increased the severity of soybean response when tank-mixed with dicamba in all site-years of the research.

Objective

In order to better understand crop response to OTD of auxin herbicides and glyphosate-auxin interaction, research was conducted to characterize the response of susceptible cotton and soybean cultivars to sub-lethal concentrations of different product

formulations of dicamba, and to determine how the presence or absence of glyphosate potassium affects crop response.

Materials and Method

Design and Treatments

Experiments were conducted from 2015 to 2018 in four locations across Mississippi for a total of seven site-years in cotton and six site-years in soybeans to evaluate the effect of glyphosate presence and dicamba formulation on crop response. Site information is shown in Table 5.1. The cotton cultivar 'DP 1321 B2RF' (Bayer Corporation) was seeded at a rate of 128,440 seeds ha^{-1}, and the soybean cultivar 'AG 4632' (Bayer Corporation) was seeded at a rate of 328,510 seeds ha^{-1} at a 2.5 cm depth in each site-year. These cultivars express glyphosate tolerance but are susceptible to auxin herbicides. A five by two factorial arrangement of treatments with four replicates and a non-treated control (NTC) was arranged in a randomized complete block design in each experiment. Experimental units consisted of four 76 cm rows in soybeans or four 97 cm rows in cotton, 12.2 m in length. Experiments were managed according to local Mississippi State University Extension Service recommendations. Experimental factors were dicamba product formulation (five levels) and glyphosate concentration (two levels). Dicamba product formulations used were: blank (no dicamba), dimethylamine salt (Banvel™, Bayer Corporation, 'DMA'), diglycolamine salt (Clarity™, BASF Corporation, 'DGA'), diglycolamine salt plus acetate (Xtendimax™ with Vaporgrip®, Bayer Corporation, 'DGAAC'), and the N,N-Bis-(3-aminopropyl) methylamine salt (Engenia™, BASF Corporation, 'BAPMA'). Fexapan™ (DuPont USA) is also available for use but is not included in this research as it contains the same product formulation as

Xtendimax™ with Vaporgrip®. Glyphosate tank-mix concentration was either 0 or 0.87 kg ae ha^{-1} as glyphosate potassium salt (Roundup Powermax II™, Bayer Corporation). Previous work has shown that bulkier glyphosate salts such as glyphosate dimethylamine or isopropylamine may help mitigate OTM when tank-mixed with auxin herbicides relative to glyphosate potassium (Havens et al. 2018) although glyphosate potassium has been used in the present study to simulate a potential worst-case OTM scenario. Herbicide application occurred at the V5 growth stage in soybeans (Fehr and Caviness 1977) and the pinhead square growth stage in cotton (Stewart et al. 1986). All dicamba was applied at 8.74 g ae ha^{-1}, a concentration that has been shown by previous research to be sub-lethal but potent enough to cause visible injury on susceptible crops (Smith et al. 2012). This dicamba concentration is also within the range commonly used to simulate off-target dicamba deposition (Egan et al. 2014; Johnson et al. 2012; Marple et al. 2007). All herbicide applications were made at 4.8 KPH using a carbon dioxide-pressurized plot backpack sprayer calibrated to deliver 140 L ha^{-1} at 276 kPa through a four-nozzle spray boom equipped with TTI11002 spray tips (TeeJet Technologies) 51 cm above the crop canopy. Herbicide applications were made to the center two rows of each plot and the outer two rows served as spray buffers. Climate and weather conditions each year at all locations fell within average historical ranges.

Data Collection and Analyses

Visible estimates of crop injury were made at weekly intervals from seven to 28 d after treatment (DAT) according to an injury scale ranging from 0 (no visible injury) to 100 (complete plant mortality) (Frans et al. 1986). Commonly observed symptoms in cotton were leaf chlorosis and curling and changes in ht. In soybeans, leaf margin cupping and

fusing, stem thickening, and node stacking was observed. Nodes above cracked-boll (NACB) counts from the center two rows of each cotton plot were recorded when the NTC reached five or less nodes above white flower (NAWF) as described by Bourland et al. (1992) and Kerby et al. (1992). Soybean node counts were recorded at defoliation from the center two rows of each soybean plot. Crop ht was recorded from the center two rows of each plot following harvest aid application. All cotton plots were harvested using a two-row plot picker, and seed cotton wt was recorded and presented here. Soybean plots were harvested using a two-row plot combine.

Cotton and soybean data were analyzed independently. Studentized residual values were calculated for each data point prior to ANOVA, and values in excess of 2.5 were removed as outliers in order to avoid outlier effects on analysis. Crop injury, node/NACB counts, crop ht, and yield data were subjected to ANOVA within the PROC MIXED feature of SAS 9.4 (SAS Institute, Inc., Cary, NC 27513). Means were separated using Fisher's protected LSD at the $\alpha = 0.05$ level of significance. A true NTC (no dicamba or glyphosate) was included in the experiment for comparison purposes but was not included in the analyses in order to minimize mean-squared error. Additional ANOVA was conducted on all parameters with or without glyphosate and with no dicamba to clarify if an effect of glyphosate presence was observed in the absence of dicamba. Whenever a two-factor interaction between dicamba formulation and glyphosate level was detected, the SLICE feature of the SAS 9.4 pdmix800 macro was utilized to conduct pairwise least squared means comparisons on the treatment combination means within PROC MIXED at the $\alpha = 0.05$ significance level (Saxton 1998). Dicamba product formulation and glyphosate concentration were analyzed as

fixed effects, and year and location combinations were combined as environments and analyzed as random effects, as were interaction effects between main effects and environment. All data met appropriate model and distribution assumptions. All data were pooled over environment in order to estimate treatment effects of a broad inference space (Blouin et al. 2011; Carmer et al. 1989). This approach utilizing multiple-environment trials is useful for making inferences over time and space and a variety of environments (Blouin et al. 2011; Carmer et al. 1989; Walker et al. 2008; Yang 2010).

Results and Discussion

Cotton

No effect from glyphosate presence without dicamba was detected for any response parameter (data not shown), which is intuitive given that the cultivar used in this research expresses tolerance to glyphosate via an engineered EPSP synthase gene that expresses an enzyme insensitive to glyphosate (Green 2007). This may imply that an effect on crop response from tank-mixing glyphosate with a dicamba formulation is due to glyphosate augmenting auxin activity as opposed to being caused by glyphosate itself (Skelton et al. 2017). Cotton ht at defoliation was only affected by dicamba formulation ($p = 0.0001$); this effect is shown averaged over glyphosate presence in Table 5.2. Exposure to dicamba DGAAC, one of the two new formulations, resulted in greater cotton ht (95 cm) than exposure to any of the other formulations, which resulted in cotton ranging from 88 to 90 cm. Visible cotton injury 28 DAT ranged from 26 to 31% and was not affected by dicamba formulation, glyphosate presence, nor any interaction thereof ($p > 0.324$). This relatively low (compared to a higher dicamba concentration or to 2,4-D) incidence of visible injury is consistent with previous findings (Buol et al. 2018; Egan et

al. 2014; Egan and Mortensen 2012; Marple et al. 2007). While 30% injured cotton may be alarming to producers, it is important to consider that in-season estimates of visible injury often overestimate potential yield losses at the end of the year and do not take into consideration the ability of crops to recover and compensate (Buol et al. 2018; Egan et al. 2014; Marple et al. 2007). Nodes above cracked boll at defoliation ranged from eight to nine and was not affected by any experimental factor or interactions (data not shown); these values are similar to NACB reported by Buol et al. (2018) following exposure to a sub-lethal concentration of dicamba diglycolamine at the pinhead square growth stage. Seed cotton yield was affected by glyphosate presence ($p < 0.0001$) and this effect is shown in Table 5.3. When averaged over dicamba formulation, the presence of a glyphosate tank-mix decreased seed cotton yield from 2,173 to 1,734 kg ha^{-1}, a reduction of approximately 20% (Table 5.3). Increased yield loss due to the addition of glyphosate is likely due to increased uptake of dicamba, similar to Skelton et al. (2017) who demonstrated increased 2,4-D uptake in corn when tank-mixed with glyphosate. The adjuvant load in the formulated glyphosate used in this study (Roundup Powermax II™) may also have contributed to the yield loss, although this is unlikely given the addition of glyphosate alone (without dicamba) did not affect yield ($p = 0.8491$).

Soybeans

Glyphosate alone did not affect any response parameter (data not shown). At the $\alpha = 0.1$ level of significance soybean yield was affected independently by both dicamba formulation ($p = 0.0515$) and glyphosate presence ($p = 0.0011$), which are shown in Tables 5.2 and 5.3, respectively. When averaged over glyphosate presence, exposure to dicamba DGA resulted in greater soybean yield (2,867 kg ha^{-1}) than exposure to dicamba

DMA (2,604 kg ha^{-1}), both of which were similar to yield following exposure to dicamba DGAAC and BAPMA. These results are consistent with previous work that reported greater severity of soybean response to dicamba DMA than dicamba DGA (Thompson et al. 2007) and the proclivity of dicamba DMA to persist in the air in greater concentrations following application (Egan and Mortensen 2012; Mueller et al. 2013). When averaged over dicamba formulation, the presence of glyphosate in spray applications led to a decrease in soybean yield from 2,872 kg ha^{-1} to 2,626 kg ha^{-1}, which is similar to the cotton yield results and may be attributed to increased dicamba uptake when tank-mixed with glyphosate (Skelton et al. 2017).

Soybean ht at maturity, visible injury 28 DAT, and node counts at maturity were each affected by an interaction between dicamba formulation and glyphosate presence (*$p < 0.0001, 0.0024, 0.0108,$ respectively*). The LS means separation following multiple comparisons of treatment combination means is shown two tables: Table 5.4 shows the effect of glyphosate level within fixed levels of dicamba formulation; and Table 5.5 shows the effect of dicamba formulation within fixed levels of glyphosate presence (present, 0.87 kg ae ha^{-1}; or absent, 0.00 kg ae ha^{-1}).

When dicamba formulation level was fixed, the addition of glyphosate did not affect node counts following exposure to any formulation except dicamba DGA, which resulted in a slight increase in node count from 11 to 13 (Table 5.4). Similarly, when glyphosate level was fixed at absent only a slight difference in soybean node counts was reported if dicamba BAPMA (12 nodes) or dicamba DGA (11 nodes) were applied (Table 5.5). When glyphosate level was fixed at present, exposure to dicamba DGA resulted in soybeans with more nodes (13) than all other dicamba formulations except

DGAAC (12). Buol et al. (2018) also reported minimal effects on cotton node counts following exposure to a sub-lethal dicamba concentration.

When dicamba formulation was fixed, the addition of glyphosate led to reduced soybean ht for all formulations except dicamba DGAAC, which resulted in soybean ht of 55 to 56 cm regardless of glyphosate presence, and DGA, which resulted in an increase in ht from 56 to 60 cm (Table 5.4). When glyphosate level was fixed at absent, exposure to dicamba BAPMA and dicamba DMA each resulted in taller soybeans (59 to 60 cm) than dicamba DGAAC or dicamba DGA (56 cm, Table 5.5). Conversely, when glyphosate level was fixed at present, exposure to dicamba DGA resulted in taller soybeans (60 cm) than any other dicamba formulation (54 to 56 cm, Table 5.5). When dicamba formulation was fixed, soybean visible injury nearly doubled from 24 to 50% when glyphosate was added to either dicamba DMA or dicamba DGA (Table 5.4), consistent with Skelton et al. (2017). When glyphosate level was fixed at absent, no differences in visible soybean injury due to dicamba formulation were observed (Table 5.5). However, when glyphosate level was fixed at present, dicamba DGAAC and BAPMA resulted in reduced injury (26 to 37%) relative to the older formulations of dicamba DMA and DGA (49 to 50%) (Table 5.5).

Conclusions

Glyphosate tends to amplify visible injury severity and morphological effects on soybean and cotton height and node counts following exposure to a sub-lethal concentration of dicamba, even in glyphosate-tolerant cultivars. This may be due to increased auxin uptake which has been reported in previous work in corn (Skelton et al. 2017). Increased sensitivity of soybeans to dicamba relative to cotton is reflected by

greater effects on visible injury and node counts, and is consistent with previous work (Egan et al. 2014; Marple et al. 2007; Thompson et al. 2007). While cotton and soybean yield losses due to dicamba formulation and/or glyphosate presence ranged from 7 to 20%, the true yield reduction would likely be considerably higher compared to a NTC, which was not included in these analyses to allow for more accurate differentiation of crop response to dicamba formulations. When considered in this specific context of OTM of dicamba onto susceptible cultivars, older dicamba formulations such as dicamba DMA appear to be more deleterious to soybeans and cotton than newer formulations such as dicamba DGAAC or BAPMA. However, distinctions between injury caused by dicamba DGA, DGAAC, and BAPMA remain difficult to make at the field level and likely require an analytic approach for differentiation.

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Tables

Table 5.1 Location, year, longitude, latitude, elevation, soil type, and planting and harvest dates for each experiment site-year.

Location	Year	Longitude	Latitude	Elevation	Soil Type[a]	Planting Date		Harvest Date	
				m		cotton	soybeans	cotton	soybeans
Brooksville	2015	88°34'W	33°15'N	85	Brooksville silty clay	21 May	15 May	21 October	14 October
Brooksville	2016	88°32'W	33°15'N	76	Okolona silty clay	26 April	-	7 October	-
Starkville	2015	88°46'W	33°28'N	81	Catalpa silty clay loam	4 May	4 May	9 September	23 September
Starkville	2016	88°46'W	33°28'N	81	Catalpa silty clay loam	26 April	-	27 September	-
Starkville	2017	88°46'W	33°27'N	84	Marietta fine sandy loam	19 May	2 May	24 October	2 October
Starkville	2018	88°46'W	33°27'N	84	Marietta fine sandy loam	1 May	1 May	21 September	25 September

Table 5.1 (continued)

West Starkville	2018	88°86'W	33°49'N	83	Leeper silty clay loam	-	10 May	-	25 September
Scott	2018	91°07'W	33°59'N	39	Robinsonville-Crevasse silty clay loam	-	23 May	-	4 October
Stoneville	2018	90°55’W	33°24’N	41	Sharkey clay	5 June	-	10 November	-

aSource: U.S. Department of Agriculture, Natural Resources Conservation Service (2018) http://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx

Table 5.2 The effect of formulation of 8.74 kg dicamba ae ha^{-1} on cotton height and soybean yield averaged over glyphosate presence following exposure to pinhead square cotton or V5/V6 soybeans.[a,b]

Formulation	Cotton Height	Soybean Yield
	(cm)	(kg ha^{-1})
DGAAC	95 a	2,750 ab
DGA	90 b	2,867 a
BAPMA	89 b	2,776 ab
DMA	88 b	2,604 b

[a] Abbreviations: DGAAC, dicamba diglycolamine plus acetate; DGA, dicamba diglycolamine; BAPMA, dicamba N,N-Bis-(3-aminopropyl) methylamine; DMA, dicamba dimethylamine
[b] Values within a column that share a letter are not different based on Fisher's protected LSD at $p \leq 0.05$

Table 5.3 The effect of tank-mixing 0.87 kg glyphosate ae ha^{-1} with dicamba on cotton and soybean yield averaged over dicamba formulations following pinhead square cotton or V5/V6 soybean exposure to 8.74 g dicamba ae ha^{-1}.[a]

Glyphosate Concentration	Cotton Yield[b]	Soybean Yield
(kg ae ha^{-1})	----------(kg ha^{-1})----------	
0.00	2,173 a	2,872 a
0.87	1,734 b	2,626 b

[a]Values within a column that share a letter are not different based on Fisher's protected LSD at $p \leq 0.05$
[b]Cotton yield is presented as machine-harvested seed cotton wt

Table 5.4 The effect of tank-mixing 0.87 kg ae glyphosate ha^{-1} with various dicamba formulations on soybean height, visible injury, and node counts following exposure of pinhead square cotton or V5/V6 soybeans to 8.74 g dicamba ae ha^{-1}. [a,b,c]

Dicamba Formulation	Glyphosate Concentration	Height	Visible Injury	Nodes
	(kg ae ha^{-1})	(cm)	(%)	(count)
BAPMA	0	60 a	21 a	12 a
	0.87	56 b	26 a	12 a
DGA	0	56 b	24 b	11 b
	0.87	60 a	50 a	13 a
DMA	0	59 a	25 b	11 a
	0.87	54 b	49 a	11 a
DGAAC	0	56 a	30 a	12 a
	0.87	55 a	37 a	12 a

[a] Abbreviations: DGAAC, dicamba diglycolamine plus acetate; DGA, dicamba diglycolamine; BAPMA, dicamba N,N-Bis-(3-aminopropyl) methylamine; DMA, dicamba dimethylamine
[b] Values within a column that share a letter are not different based on Fisher's protected LSD at $p \leq 0.05$
[c] Values within a column are to be compared within the same dicamba formulation, denoted by each subsection bounded by dashed lines

Table 5.5 The effect of formulation of 8.74 g dicamba ae ha^{-1} with or without 0.87 kg glyphosate ae ha^{-1} on soybean height, visible injury, and node counts following exposure at the V5/V6 growth stage. [a,b]

Glyphosate Concentration	Dicamba Formulation	Height	Visible Injury	Nodes[c]
(kg ae ha^{-1})		(cm)	(%)	(count)
0	BAPMA	60 a	21 a	12 a
	DMA	59 a	25 a	11 ab
	DGAAC	56 b	30 a	12 ab
	DGA	56 b	24 a	11 b
0.87	BAPMA	56 b	26 b	12 b
	DMA	54 b	49 a	11 b
	DGAAC	55 b	37 b	12 ab
	DGA	60 a	50 a	13 a

[a] Abbreviations: DGAAC, dicamba diglycolamine plus acetate; DGA, dicamba diglycolamine; BAPMA, dicamba N,N-Bis-(3-aminopropyl) methylamine; DMA, dicamba dimethylamine
[b] Values within a column that share a letter are not different based on Fisher's protected LSD at $p \leq 0.05$
[c] Node counts are generated as estimates by PROC MIXED and then rounded to the nearest whole number, resulting in some cases where two of the same values may have different mean separation groupings due to whether they have been rounded up or down to the presented value
[d] Values within a column are to be compared within the same glyphosate concentration, denoted by each subsection bounded by dashed lines

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CHAPTER VI

OPTIMIZING CHLOROACETAMIDE APPLICATION TIMING IN DICAMBA-RESISTANT SOYBEAN PRODUCTION SYSTEMS FOR CONTROL OF GLYPHOSATE-RESISTANT *AMARANTHUS SPP*. AND KOCHIA (*BASSIA SCOPARIA* L.)

Abstract

Proper herbicide stewardship and resistance management will be imperative as producers continue to adopt the dicamba-resistant technology in the Xtend™ Weed Control System. One component of resistance management is diversifying herbicide use in weed control. Chloroacetamide herbicides effectively control small-seeded broadleaf weeds and are labeled for flexible application in soybean production systems. A chloroacetamide herbicide by application timing factorial experiment was conducted in 2017 and 2018 in Mississippi and Nebraska to optimize chloroacetamide use in dicamba-based Palmer amaranth, waterhemp, and kochia management systems in soybean production. Herbicides used were *s*-metolachlor and acetochlor, and application timings were PRE, PRE fb EP, PRE fb LP, EP alone, LP alone, and EP fb LP. Differences in response due to chloroacetamide type were minimal, and soybean injury following any application timing was under 5%. Late-season visible weed control was reduced by 8 to 31% if a single chloroacetamide application was used as opposed to a split application. Late-season weed densities were minimized by any application timing other than PRE alone. Soybean heights varied based upon application timing and herbicide. Chloroacetamide applications at any timing except PRE alone maximized weed biomass reduction and soybean

yield. While no yield loss was reported by delaying the first application (of a split application) until EP, foregoing a PRE application in hopes of timely POST applications is not advisable given the multiple factors that may delay timely POST applications, as well as the increased chance for higher late-season weed densities increasing selection pressure on POST herbicides.

Nomenclature: Acetochlor; dicamba; glyphosate; kochia, *Bassia scoparia* (L.) A.J. Scott; Palmer amaranth, *Amaranthus palmeri* S. Watson; *s*-metolachlor; soybean, *Glycine max* (L.) Merr.; waterhemp, *Amaranthus tuberculatus* (Moq.) Sauer

Key words: Acetochlor, chloroacetamide, soybean, dicamba, *s*-metolachlor

Introduction

Soybean (*Glycine max* (L.) Merr.) cultivars available for use in the Roundup Ready® XTEND Crop System (Bayer Corporation, Whippany, NJ 07981) contain engineered resistance to dicamba and glyphosate (Behrens et al. 2007; Feng and Brinker 2014). The adoption of these cultivars has been widespread, with approximately 8.1 million ha domestically being planted to XTEND® soybeans in 2017; with up to 60% of soybean ha in some areas (Lingenfelter et al. 2017). Adoption of this technology is high and expected to rise due to producers' desire to control herbicide-resistant (HR) weeds, utilize elite crop germplasm, and defend crops against off target dicamba deposition (Egan et al. 2014; Mortensen et al. 2012). Dicamba-based weed control programs are especially promising given the relatively low incidence of dicamba-resistant weed species (Heap 2019).

Palmer amaranth constitutes the greatest weed threat to producers in the South, and is one of the most problematic weeds in the U.S. (Webster 2012, 2013). Waterhemp (*Amaranthus tuberculatus* (Moq.) Sauer) is increasingly difficult to discern from Palmer amaranth due to continued hybridization between the species (Heap 2019; Meyer et al. 2015; Steckel 2007), and

thus may be considered together with Palmer amaranth when discussing the deleterious nature of *Amaranthus* spp. domestically. Several characteristics of Palmer amaranth provide competitive advantages over soybeans such as rapid growth, large leaf and plant structures, and high fecundity with long durations of reproduction (Culpepper et al. 2010; Horak and Loughin 2000; Sellers et al. 2003; Webster and Grey 2015). Palmer amaranth densities of one plant per 0.125 m of row can reduce soybean yield by up to 78% (Bensch et al. 2003). Similarly, Klingaman and Oliver (1994) reported soybean yield reductions ranging from 17 to 68% from Palmer amaranth densities of 0.33 to 10 plants m^{-1} of row, respectively. Palmer amaranth with multiple resistance to glyphosate and acetolactate synthase (ALS) inhibitors has led to a shift to alternative herbicide modes-of-action (MOAs), such as glufosinate and protoporphyrinogen oxidase (PPO) inhibitors (Heap 2019; Horak and Peterson 1995; Miller and Norsworthy 2016; Sosnoskie and Culpepper 2014; Sprague et al. 1997). Palmer amaranth populations with resistance to PPO inhibitors have already been reported as a result of this shift (Heap 2019; Salas et al. 2016). Palmer amaranth with multiple-resistance to many herbicide MOAs is a widespread problem, especially populations with resistance to ALS inhibitors and glyphosate (Bagavathiannan and Norsworthy 2013).

Kochia is a serious concern in crop production in the Great Plains, the western U.S. and Canada, and is also present in the Eastern U.S. (Crespo et al. 2014; Eberlein and Fore 1984; Forcella 1985; Stubbendieck et al. 2003). Kochia is highly competitive due to high levels of genetic variation, cross-pollination, early and rapid emergence, drought tolerance, and many dispersal vectors including physically moving across the landscape as a ‘tumbleweed’ (Crespo et al. 2014; Durgan et al. 1990; Mengistu and Messersmith 2002; Pafford and Wiese 1964; Schwinghamer and Van Acker 2008). Additional concern arises when considering that although

there is a low relative incidence of dicamba-resistant weeds overall (Heap 2019), there have been several reports of kochia populations with reduced or no dicamba sensitivity in several states of the Midwestern and Western U.S. (Cranston et al. 2001; Crespo et al. 2014; Preston et al. 2009), and that 32 to 52% of Nebraska survey respondents rated kochia as having a high to medium chance of developing resistance to kochia (Crespo et al. 2012). Kochia has been reported to establish densities over 2,100 plants m^{-2}, reduce soybean yield by over 30%, and interfere with soybean harvest (Wolf et al. 2000). In addition to soybeans, kochia is also problematic in wheat (*Triticum aestivum* L.), sugar beet (*Beta vulgaris* L.), corn (*Zea mays* L.), sorghum (*Sorghum bicolor* (L.) Moench), and sunflower *(Helianthus annuus* L.), where it has been reported to reduce crop yields by up to 95% (Durgan et al. 1990; Kumar et al. 2014; Mesbah et al. 1994; Weatherspoon and Schweizer 1971; Wicks et al. 1993, 1994).

Resistance management has been advocated for some time, but producers continue to rely on reactive vs proactive strategies implies a cessation in development of herbicide-resistant weeds is unlikely (Meyer et al. 2015; Heap 2019). Despite the relatively low current incidence of dicamba-resistant weeds, Neve et al. (2011) reported that only 4,000 Palmer amaranth plants producing 250,000 seeds $plant^{-1}$ would be necessary to produce five seeds with herbicide resistance given a mutation rate of five per one billion individuals. Palmer amaranth and kochia demonstrate high fecundity and genetic variation and are capable of reproducing in such numbers (Burke et al. 2007; Crespo et al. 2012, 2014; Keeley et al. 1987; Massinga et al. 2001; Webster and Grey 2015). In order to preserve dicamba-resistant weed control technology, weed control strategies with a proactive focus on resistance prevention will be important moving forward (Norsworthy et al. 2012). The use of herbicides with multiple MOA tank-mixed and in rotation has been shown to delay the development of herbicide resistance by up to four years as

opposed to rotating herbicides every other year (Beckie and Reboud 2009; Gressel and Segel 1990; Powles et al. 1997; Wrubel and Gressel 1994). Given a 100 ha area, a beginning seed bank of 100 seeds m^2, and a resistant gene frequency of one in ten million, Neve et al. (2003) reported resistance developing to each of two herbicides used as a tank-mix was unlikely within 50 years. When the herbicides were rotated in alternating years, however, multiple resistance to each herbicide arose almost ubiquitously (Neve et al. 2003).

Combining glyphosate and glufosinate with 2,4-D or dicamba has been shown to effectively control glyphosate-resistant (GR) Palmer amaranth across multiple densities and growth stages (Cahoon et al. 2015b; Chahal and Johnson 2012; Merchant et al. 2013; Merchant et al. 2014; Montgomery et al. 2017; Vann et al. 2017). It is important to note that while both glyphosate and dicamba are labeled for use in XTEND® soybeans, many areas utilizing this technology likely already contain GR weeds, and POST applications of glyphosate plus dicamba provides only one effective herbicide MOA (dicamba). Recommendations for the inclusion of residual herbicides in weed management plans focused on sustainably controlling GR weeds are common (Culpepper et al. 2009; Everman et al. 2009; Gardner et al. 2006; Sosnoskie and Culpepper 2014). The Group 15 (long-chain fatty acid inhibitors) chloroacetamide herbicides *s*-metolachlor and acetochlor are tank-mix candidates with dicamba for residual control of GR weeds. These herbicides prevent weed emergence, are safe on soybeans, and are labeled for flexible application timing. Braswell et al. (2016) reported that acetochlor or fluridone could be utilized to reduce selection pressure on PPO-inhibiting herbicides in Palmer amaranth management programs.

S-metolachlor and acetochlor are generally safe on crops. Cahoon et al. (2015a) reported that microencapsulated acetochlor (Warrant® Herbicide, Bayer Corporation) caused 3 to 8% less

injury to cotton than pendimethalin. However, crop safety has been a concern when with *s*-metolachlor due to its potential to injure cotton following PRE use (Brown et al. 1993; Keeling and Abernathy 1989). Stephenson et al. (2013) reported 7% cotton injury following application of *s*-metolachlor alone to two- to three- lf cotton, and up to 31% injury if *s*-metolachlor was co-applied with pyrithiobac, although this injury was transient. When considering *s*-metolachlor coapplied with glyphosate, Clewis et al. (2006) reported 3% cotton injury following application of *s*-metolachlor alone or co-applied with either of two glyphosate formulations. However, this 3% increase in injury due to co-application of *s*-metolachlor with glyphosate corresponded with 6 to 46% greater Palmer amaranth control (Clewis et al. 2006).

Meyer et al. (2015) reported up to 94% control of GR Palmer amaranth in soybeans following synthetic auxin application early postemergence (EP), compared to increased weed pressure if application was delayed until late postemergence (LP). However, use of a synthetic auxin LP provided greater overall control than treatments that did not include a LP application, and more herbicide sites of action (SOA) utilized generally resulted in greater weed control (Meyer et al. 2015). A split chloroacetamide application (dicamba plus acetochlor PRE fb *s*-metolachlor plus glyphosate plus dicamba POST) resulted in 89% control of GR weeds in soybean 28 d after LP (DALP) (Meyer et al. 2015). Cahoon et al. (2015a) found Palmer amaranth control prior to POST glufosinate application was 20% greater following PRE use of acetochlor compared to pendimethalin, and was greater than control from diuron, fluometuron, or fomesafen. Steele et al. (2005) observed over 91% early-season control of Palmer amaranth following PRE application of *s*-metolachlor. Geier et al. (2006) reported up to 100% Palmer amaranth control by *s*-metolachlor. Similarly, Whitaker et al. (2010) reported increased season-long Palmer amaranth control (87, 77, and 63% at 0, 30, and 90 d after POST application,

respectively) and soybean yield following PRE use of *s*-metolachlor relative to pendimethalin. Likewise, increased kochia control has repeatedly been reported following co-application of dicamba with a residual herbicide relative to dicamba alone (Kumar and Jha 2015; Nandula and Manthey 2002; Wicks et al. 1994).

Objective

In order to determine the optimal application timing and chloroacetamide herbicide for GR Palmer amaranth and kochia management in dicamba-based soybean production, research was conducted to evaluate weed and crop response to acetochlor and s-metolachlor applied at six different timings.

Materials and Method

Design and Treatment

Field research was conducted at Brule, Nebraska; Robinsonville, Mississippi; and Starkville, Mississippi in 2017 and 2018 to determine optimal use of chloroacetamide herbicides for GR Palmer amaranth, waterhemp, and kochia management in dicamba-resistant soybean production systems of the southern and Midwest United States. Six total site-years of research were conducted, and site information is shown in Table 6.1. Each site contained a native population of GR Palmer amaranth, waterhemp, or kochia, with densities shown in Table 6.2. The soybean variety 'AG46X6' (ASGROW®, Bayer Corporation, resistance to glyphosate and dicamba) was seeded at 321,100 seeds ha^{-1} at a 2.5 cm depth at all Mississippi site-years. The soybean variety 'AG24X7' (ASGROW®, Bayer Corporation, resistance to glyphosate and dicamba) was seeded at 333,450 seeds ha^{-1} at a 2.5 cm depth at each Brule, Nebraska site-year. Experiments were arranged in a two by six factorial arrangement of treatments in a randomized complete block.

Each site-year contained four replications and a non-treated control (NTC) for comparison purposes. Experimental units measured 12.2 m in length and consisted of four 97 cm rows. Experiments were managed according to local production recommendations from the Mississippi State University Extension Service (Anonymous 2018) and the University of Nebraska Extension Service (Anonymous 2019). This research was conducted under dryland production conditions in Mississippi. Research at Brule, NE utilized overhead irrigation delivering 6.35 to 12.7 mm of precipitation each week as necessary.

Chloroacetamide herbicide (two levels) and herbicide application timing (six levels) were the experimental factors. Chloroacetamide herbicide levels consisted of *s*-metolachlor (Dual Magnum®, Syngenta US, Greensboro, NC 27419) and acetochlor (Warrant®, Bayer Corporation). Application timing levels consisted of: (1) PRE immediately following planting, (2) PRE followed by (fb) early POST [PRE fb EP] at V2 soybean (Fehr and Caviness 1977), (3) PRE fb late POST [PRE fb LP] at V5-V6 soybean (Fehr and Caviness 1977), (4) EP alone, (5) LP alone, and (6) split POST [EP fb LP]. Herbicide application rates were calculated based upon the maximum labeled application rate for *s*-metolachlor (the more restrictive of the two labels). Based on these calculations, *s*-metolachlor was applied PRE, EP, or LP alone at 1.42 kg ai ha^{-1}, and acetochlor applied at these timings was applied at 1.27 kg ai ha^{-1}. Due to the maximum limit of 1.42 kg ai *s*-metolachlor ha^{-1} labeled for POST use, a concentration of 0.71 kg ai ha^{-1} *s*-metolachlor was utilized for the split POST application treatment (EP fb LP). Similarly, acetochlor was applied at a rate of 0.64 kg ai ha^{-1} per application for the split POST application treatment. The treatment combinations utilized in this experiment are shown in Table 6.3 along with herbicide rate and soybean stage at application. This research is meant to analyze chloroacetamide use as one part of a more comprehensive, dicamba-based weed control system.

As such, the maximum labeled rates of 0.56 kg ae ha^{-1} dicamba (XTENDIMAX®, Bayer Corporation) was included with all PRE applications, and 0.77 kg glyphosate ae ha^{-1} plus 0.39 kg dicamba ae ha^{-1} (Roundup XTEND™ with VaporGrip™, Bayer Corporation) was included with all POST applications. Herbicide applications were made with a carbon dioxide-pressurized plot backpack sprayer operated at 4.8 KPH and calibrated to deliver 140 L ha^{-1} at 276 kPa. A spray boom equipped with TTI11002 spray tips (TTI, TurboTee Induction, TeeJet Technologies, Glendale Heights, IL 60139) was operated 51 cm above crop canopy. All herbicide applications were made to the center two rows of each four-row plot and the outer two rows served as buffers between plots. Heat and precipitation accumulation varied by year, but annual totals fell within average historical ranges at each site year and are shown in Table 6.2. Precipitation accumulation values for Brule, NE include irrigation totals, and no irrigation was utilized at Mississippi sites (Table 6.2).

Data Collection and Analyses

Estimates of visible crop injury and weed control were made weekly from seven to 28 d after each treatment (DAT) and ranged from zero (no visible injury) to 100 (complete plant mortality) (Frans et al. 1986). Weed density was recorded by counting all live plants in a randomly selected 0.25 m^2 quadrat section of each plot 28 d after each application. Late-season weed biomass was harvested from this same 0.25 m^2 section following weed density collection 28 d after the LP application. Weed biomass was harvested by cutting all plants in the 0.25 m^2 quadrat section at ground level and collecting them for drying. Fresh biomass samples were then placed in a 40° C forced-air oven for 72 h and dry biomass was recorded. Paraquat (Gramoxone® SL 2.0, Syngenta) was applied at 0.42 kg cation ha^{-1} as a harvest aid when 90%< soybeans reached full maturity. Soybean ht at maturity was recorded from the center two rows of each plot

by randomly selecting six plants in each of the two center rows. Soybean yield was harvested using a two-row plot combine.

Data were analyzed with SAS 9.4 (SAS Institute, Inc., Cary, NC 27513). Studentized residuals were calculated for data points of each response parameter, and those in excess of 2.5 were removed as outliers prior to ANOVA in order to avoid outlier effects on analysis. Studentized residual analysis was also used to assess and confirm variance homoscedasticity. Soybean injury, ht, and yield and visible weed control, weed density and biomass data were each subjected to ANOVA using the SAS 9.4 PROC MIXED procedure, and means were separated using Fisher's protected LSD at the $\alpha = 0.05$ level of significance. The NTC was not included in the analyses in order to minimize MSE and allow greater mean separation between response parameter means. The SLICE feature of the SAS 9.4 pdmix800 macro was utilized to conduct pairwise least squared means comparisons within PROC MIXED at the $\alpha = 0.05$ significance level (Saxton 1998). Year and location were combined as environment and considered a random effect given it affected the magnitude of mean responses but not any trends in response. Accordingly, chloroacetamide herbicide and application timing were analyzed as fixed effects and environment as a random effect. All data were thus pooled over environment in order to estimate treatment effects over a wide variety of environments (Blouin et al. 2011; Carmer et al. 1989). This approach allows broad inferences over a range of time and space (Blouin et al. 2011; Carmer et al. 1989; Walker et al. 2008; Yang 2010). At the Brule, NE location in late June 2018, the entire experimental area suffered widespread soybean mortality following hail and wind damage from a severe storm. Thus, no soybean data (injury, height, and yield) is included from this location beginning July 1, 2018; and all weed data (control, density, biomass) after this date are based upon applications made in bareground conditions.

Results and Discussion

Evaluations 28 d After PRE

Weed control 28 d after PRE (DAPRE) was affected by chloroacetamide application timing ($p < 0.0001$). Table 6.4 shows the effect of chloroacetamide application timing on weed control 28 d after PRE, EP, and LP; and on soybean injury 28 d after PRE and LP, averaged over chloroacetamide herbicide. Visible weed control 28 DAPRE ranged from 63 to 100% (Table 6.4), which is within the range of early-season weed control reported by previous research investigating chloroacetamide control of GR Palmer amaranth (Cahoon et al. 2015a; Geier et al. 2006; Kumar and Jha 2015; Myer et al. 2015; Steele et al. 2005). Delaying initial chloroacetamide application until POST resulted in up to a 37% reduction in weed control 28 DAPRE, whereas use of a chloroacetamide PRE alone or fb POST resulted in 97 to 100% weed control (Table 6.4), consistent with previous research that reported increased weed control following applications PRE and POST relative to POST only (Cahoon et al. 2015a; Meyer et al. 2015). Soybean injury 28 DAPRE was affected by chloroacetamide herbicide ($p < 0.0001$, Table 6.5). *S*-metolachlor was slightly more injurious to soybeans 28 DAPRE than acetochlor, though injury was minor following application of either herbicide (5% and 2%, respectively). This low magnitude of crop injury, with slightly more injury following use of *s*-metolachlor relative to acetochlor, is consistent with previous findings (Cahoon et al. 2015a, 2015b; Everman et al. 2009; Stephenson et al. 2013). A chloroacetamide herbicide by application timing interaction was detected as affecting weed density 28 DAPRE ($p = 0.0039$) and is shown in Table 6.6. Weed densities (averaged over Palmer amaranth, waterhemp, and kochia) ranged from 10 to 166 plants m^{-2} (Table 6.6). Weed density 28 DAPRE was minimized by use of any treatment containing *s*-metolachlor PRE (PRE, PRE fb EP, PRE fb LP) or *s*-metolachlor EP. While the use of *s*-

metolachlor EP resulted in similar weed density 28 DAPRE, delaying initial acetochlor application until POST did result in a numerical increase in weed density (Table 6.6). Acetochlor did not reduce weed density as effectively as *s*-metolachlor following any application timing except EP fb LP, which was similar to *s*-metolachlor EP fb LP (64 and 166 plants m^{-2}, respectively). Previous research supports the conclusion that co-application of a residual herbicide with a POST herbicide such as dicamba results in increased control of GR weeds and reduced weed densities relative to dicamba alone (Geier et al. 2006; Kumar and Jha 2015; Steele et al. 2005; Whitaker et al. 2010).

Evaluations 28 d After EP

Weed control evaluated 28 d after EP (DAEP) was affected by chloroacetamide application timing ($p < 0.0001$). Control ranged from 80 to 97% when averaged over chloroacetamide application timing (Table 6.4), similar to the range of weed control reported in previous research (Cahoon et al. 2015a; Meyer et al. 2015). Control 28 DAEP was maximized (93 to 97%) by any split chloroacetamide application or application EP alone (Table 6.4). Chloroacetamide application PRE alone resulted in the minimum level of weed control 28 DAEP, although 80% control at this point (up to 60 days following planting, Table 6.1) reflects the efficacious residual nature of chloroacetamide herbicides. Delaying initial chloroacetamide application until POST resulted in either a numeric (EP alone) or statistical (LP alone) reduction in weed control relative to split applications utilizing a PRE application (Table 6.4), which is in agreeance with previous findings that including a PRE application of a residual herbicide leads to improved weed control (Culpepper et al. 2009; Everman et al. 2009; Gardner et al. 2006; Sosnoskie and Culpepper 2014). However, use of a split POST (EP fb LP) chloroacetamide application provided similar weed control 28 DAEP (97%) relative to EP or LP alone (93 and

89% respectively), which likely testifies to the ability of multiple POST applications of dicamba to control emerged weeds (Cahoon et al. 2015b). Soybean injury 28 DAEP ranged from 1 to 2% and was not affected by any main effect or interaction thereof (data not shown). Similarly, weed density 28 DAEP ranged from 5 to 15 plants m^{-2}, but was not affected by any treatments (data not shown).

Late Season Evaluations

Weed control 28 d after LP (DALP) was affected by chloroacetamide application timing ($p < 0.0001$) and ranged from 66 to 97% (Table 6.4). The greatest control 28 DALP (94 to 97%) was achieved following chloroacetamide application PRE fb EP, PRE fb LP, LP alone, or EP fb LP (Table 6.4). Chloroacetamide application EP alone provided more control 28 DALP than PRE alone (89 and 66%, respectively) although this level of control would likely be considered unacceptable on a commercial basis. However, from the standpoint of resistance management, the 66% control provided by the PRE alone (relative to a NTC) still provides considerable reduction in weed pressure and thus selection pressure on POST herbicides that may be required in a rescue scenario (Merchant et al. 2014; Vann et al 2017). Soybean injury 28 DALP was affected by chloroacetamide application timing ($p = 0.0019$) and chloroacetamide herbicide ($p = 0.0151$). When averaged over chloroacetamide application timing, soybean injury was minor and ranged from 1 to 2% (Table 6.5). Acetochlor caused 1% soybean injury 28 DALP, half as much as *s*-metolachlor (2%). However, such a minor difference in crop injury and overall injury magnitude is unlikely to affect yield and is likely impossible to consistently differentiate at the field level. Conversely, when averaged over chloroacetamide herbicide, injury 28 DALP ranged from 0 to 4% (Table 6.4). When averaged over chloroacetamide herbicide, applications EP fb LP or PRE fb LP resulted in the greatest magnitude of soybean injury (4-5%), which is intuitive

given that these treatments involved a more recent application (LP) than EP alone (2% injury), and utilized an additional application timing (PRE) relative to the LP alone timing (2% injury). The low magnitude and transient nature of crop injury is consistent with previous findings (Cahoon et al. 2015a; Clewis et al. 2006; Stephenson et al. 2013). Furthermore, application PRE alone or PRE fb EP resulted in no observable injury 28 DALP. Weed density 28 DALP ranged from 5 to 17 plants m^{-2} and was affected by chloroacetamide application timing ($p = 0.0319$, Table 6.7). No application timing achieved weed-free densities, although the majority of weeds present in density counts 28 DALP were at the cotyledon or small seedling stage, and vastly reduced compared to the NTC (author's personal observation). The minimum weed density 28 DALP was achieved by any split chloroacetamide application or applications EP or LP alone (Table 6.7). It is also important to note that all POST applications in this research included glyphosate plus dicamba, and Cahoon et al. (2015a, 2015b) reported increased control of GR Palmer amaranth with multiple POST applications of dicamba relative to a single application, so increased dicamba efficacy following multiple applications also likely plays a role in improving weed control. However, dicamba and glyphosate have little to no residual activity and therefore any differences in late season weed densities may ostensibly be attributed to chloroacetamide activity. While PRE alone resulted in the greatest weed density 28 DALP (17 plants m^{-2}), it still provided considerable reduction relative to the NTC (data not shown), thus reducing selection pressure on POST herbicides that would be needed in a rescue situation (Merchant et al. 2014; Vann et al. 2017). Weed dry biomass at soybean maturity ranged from 9 to 130 g m^{-2} and was affected by chloroacetamide application timing ($p = 0.0002$, Table 6.7). Weed biomass was minimized by any chloroacetamide application timing besides PRE alone (130 g m^{-2}). However, delaying initial application until POST led to a numeric increase in late season biomass relative

to either split application that included the use of a PRE application (PRE fb EP, PRE fb LP). Similar to weed density 28 DALP, the use of a split POST (EP fb LP) application was unable to numerically reduce late season weed biomass to values following split application (41 vs 9 to 18 g m^{-2}), implying that while a split POST application may be adequate for achieving maximum visible weed control, density and biomass reduction in such situations will likely reflect increased weed pressure, especially in the case of more severe infestations and/or situations where a timely POST application cannot be made (Merchant et al. 2014; Vann et al. 2017). Soybean ht 28 DALP was affected by an interaction between chloroacetamide type and application timing ($p < 0.0001$). Soybean ht 28 DALP ranged from 79 to 90 cm (Table 6.6). Soybean ht was maximized by use of acetochlor LP alone or *s*-metolachlor PRE alone or fb EP. Soybean ht was minimized following use of *s*-metolachlor PRE fb LP, LP alone, or EP fb LP (Table 6.6). The maximum ht differential was observed between plots treated with acetochlor LP (90cm) and those treated with *s*-metolachlor PRE fb LP, LP alone, or EP fb LP (79 to 81cm). The reduction in ht following *s*-metolachlor application at these timings may be due to the slightly increased crop injury potential of *s*-metolachlor (Cahoon et al. 2015a, 2015b; Stephenson et al. 2013; Table 6.5). However, the ambiguous numeric trend in ht as initial application (in a split application) is delayed until POST or as the second of a split application is delayed to LP is likely indicative of the conflating effects of crop injury from more recent herbicide applications (in the case of LP applications) vs. increased late-season duration of crop-weed competition following earlier application of the final (of a split) chloroacetamide application (in the case of any application ending at EP or earlier). Soybean yield ranging from 2,017 to 2,560 kg ha^{-1} was affected by chloroacetamide application timing ($p = 0.0095$), and is shown averaged over chloroacetamide herbicide in Table 6.7. All chloroacetamide application timings besides PRE

alone (2,017 kg ha^{-1}) resulted in similar yields (2,367 to 2,560 kg ha^{-1}), implying that yield was maximized by any split or POST alone use of chloroacetamide herbicides. While yield was not sensitive to differences in weed density and control following different application timings, the greater consideration of herbicide resistance management depends on mitigating weed density and pressure throughout the growing season (Culpepper et al. 2009; Everman et al. 2009; Gardner et al. 2006; Meyer et al. 2015; Norsworthy et al. 2012; Sosnoskie and Culpepper 2014).

Significance of Findings for Soybean Management

Stewardship of new dicamba-resistant cultivars and their associated weed control technology will be integral moving forward as the number of herbicide-resistant weed species continues to grow (Heap 2019). Diversifying the application timing (PRE, POST) and type (MOA) of herbicides utilized in comprehensive weed control programs has repeatedly been shown to be a key component of herbicide and resistance stewardship (Beckie and Reboud 2009; Culpepper et al. 2009; Everman et al. 2009; Gardner et al. 2006; Gressel and Segel 1990; Powles et al. 1997; Sosnoskie and Culpepper 2014; Wrubel and Gressel 1994). This research sought to identify the most impactful way to utilize one component of a herbicide system (chloroacetamides) as part of a more comprehensive dicamba-based weed control plan. Chloroacetamides such as acetochlor and *s*-metolachlor provide good residual control of GR Palmer amaranth, waterhemp, and kochia. In order to reduce weed competition with soybeans and selection pressure on POST herbicides, a split application of either *s*-metolachlor or acetochlor is advisable, preferably with one application occurring PRE. Foregoing a PRE application in favor of a split POST application may not adversely affect yields, but will likely lead to increased late-season weed density and biomass, which may translate to yield loss in more severe infestations. Furthermore, increased late-season weed density and biomass may

impede mechanical harvest (Morgan et al. 2001; Smith et al. 2000; Wolf et al. 2000). Perhaps most importantly, delaying initial chloroacetamide application until POST burdens POST herbicides such as dicamba and glyphosate with additional selection pressure due to increased early- and mid-season weed densities. A split application of either *s*-metolachlor or acetochlor PRE fb EP or LP is recommended as part of a more comprehensive dicamba-based GR weed management program. If a PRE application of these herbicides cannot be made, multiple POST applications may offer effective recourse, though this is not recommended due to the high chance for untimely POST applications and added selection pressure on POST herbicides.

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Tables

Table 6.1 Location, year, longitude, latitude, elevation, soil type, and planting, application, and harvest dates for each experiment site-year.[a]

Location	Year	Longitude	Latitude	Elevatio	Soil Type[b]	Application Date			Harvest Date
				m		PRE[c]	EP[d]	LP[e]	
Brule, NE	2017	102° 04’	41° 16’ N	1,003	Kuma loam	May	June	July 11	September 29
Brule, NE	2018	102° 04’	41° 16’ N	1,003	Kuma loam	May	July	July	--[*]
Starkville, MS	2017	88° 86' W	33° 49' N	83	Leeper silty clay	May	June 2	June 8	October 3
Starkville, MS	2018	88° 86' W	33° 49' N	83	Leeper silty clay	May	June 5	June	September 19
Robinsonville,	2017	90° 29’	34° 83’ N	60	Commerce silt	April	May	May	October 1
Robinsonville,	2018	90° 29’	34° 83’ N	60	Commerce silt	April	May	May	September 27
Robinsonville,	2018	90° 29’	34° 83’ N	60	Commerce silt	April	May	May	September 27

[a]Abbreviations: PRE, preemergence; EP, early postemergence; LP, late postemergence
[b]Source: U.S. Department of Agriculture, Natural Resources Conservation Service (2019) http://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx
[c]PRE applications occurred immediately after planting on the same day
[d]EP applications were made to soybeans at the V2-V3 growth stage
[e]LP applications were made to soybeans at the V5-V6 growth stage
[*]The 2018 Brule, NE location suffered fatal hail and wind damage following a severe storm in late June, 2018 resulting in location-wide soybean mortality. Thus, these applications were made in bareground conditions and no harvest occurred

Table 6.2 Native weed density and precipitation and heat accumulation totals 14 days after each application and season-long at each site year.[a]

Location	Year	Weed[b] density	14 DA-PRE[c]		14 DA-EP[d]		14 DA-LP[e]		Total[f]	
			Precipitation	DD60	Precipitation	DD60	Precipitation	DD60	Precipitation	DD60
		plants m^{-2}	cm		cm		cm		cm	
Brule, NE*	2017	11-22	3.18	69	3.97	213	1.50	270	38.0	1391
Brule, NE*	2018	11-22	7.70	147	8.10	239	5.11	184	--†	--†
Starkville	2017	80-90	0.25	152	0.51	196	0.38	216	11.5	2250
Starkville	2018	80-90	0.36	221	0.46	282	0.03	282	4.16	2597
Robinsonville	2017	100-150	2.31	82	12.0	176	7.98	204	36.0	2357
Robinsonville	2018	100-150	0.33	218	4.55	266	0.10	293	25.4	2609

[a]Abbreviations: 14 DA-PRE, fourteen days after preemergence application made immediately following planting; 14 DA-EP, fourteen days after early postemergence application to V2-V3 soybeans; 14 DA-LP, fourteen days after late postemergence application to V5-V6 soybeans; DD60, growing degree days (calculated by subtracting 60 from the average daily temperature)

[b]Weed species were kochia at Brule, NE; Palmer amaranth at Robinsonville, MS; and waterhemp at Starkville, MS

[c]Precipitation and DD60 data in this section are cumulative totals from the 14 days immediately following PRE herbicide application

[d]Precipitation and DD60 data in this section are cumulative totals from the 14 days immediately following EP herbicide application

[e]Precipitation and DD60 data in this section are cumulative totals from the 14 days immediately following LP herbicide application

[f]Precipitation and DD60 data in this section are cumulative totals from the entire growing season between the PRE and harvest dates shown in Table 6.1

*Precipitation totals for Brule include overhead irrigation totals

†The 2018 Brule, NE location suffered fatal hail and wind damage following a severe storm in late June, 2018 resulting in location-wide soybean mortality. Thus, no harvest date was available for calculating total precipitation or DD60 for the season.

Table 6.3 Concentrations of acetochlor or *s*-metolachlor applied PRE, EP, or LP immediately after planting, to V2 soybeans, or to V5 soybeans, respectively.[a]

Herbicide	Treatment Combination	Application Timing PRE[b]	EP[c]	LP[c]
s-metolachlor (Syngenta)	1	1.42 kg ai ha^{-1}	--	--
	2	1.42 kg ai ha^{-1}	1.42 kg ai ha^{-1}	--
	3	1.42 kg ai ha^{-1}	--	1.42 kg ai ha^{-1}
	4	--	1.42 kg ai ha^{-1}	--
	5	--	--	1.42 kg ai ha^{-1}
	6	--	0.71 kg ai ha^{-1}	0.71 kg ai ha^{-1}
Acetochlor (Bayer Corporation)	7	1.27 kg ai ha^{-1}	--	--
	8	1.27 kg ai ha^{-1}	1.27 kg ai ha^{-1}	--
	9	1.27 kg ai ha^{-1}	--	1.27 kg ai ha^{-1}
	10	--	1.27 kg ai ha^{-1}	--
	11	--	--	1.27 kg ai ha^{-1}
	12	--	0.64 kg ai ha^{-1}	0.64 kg ai ha^{-1}

[a]Abbreviations: PRE, preemergence to immediately following planting; EP, early postemergence to V2-V3 soybeans; LP, late postemergence to V5-V6 soybeans

[b]All PRE applications included 0.56 kg ae ha^{-1} dicamba (XTENDIMAX®, Bayer Corporation) made immediately following planting

[c]All POST applications included 0.77 kg glyphosate ae ha^{-1} plus 0.39 kg dicamba ae ha^{-1} (Roundup XTEND™, Bayer Corporation)

Table 6.4 Effect of chloroacetamide application timing on weed control pooled over kochia, waterhemp, and Palmer amaranth data 28 d after PRE, EP, and LP; and on soybean injury 28 d after PRE and LP, averaged over chloroacetamide herbicide.[a,b]

Application Timing	Control 28 DAPRE	Control 28 DAEP	Control 28 DALP	Injury 28 DALP
	----------%----------			
PRE	100a	80c	66c	0c
PRE fb EP	96a	97a	97a	0c
PRE fb LP	97a	96ab	94ab	4ab
EP	68b	93ab	89b	2bc
LP[c]	--	89b	95ab	2bc
EP fb LP	63b	97a	97a	5a

[a]Abbreviations: DAPRE, days after preemergence (PRE) application immediately following planting; DAEP, days after early postemergence (EP) application to V2-V3 soybeans; DALP, days after late postemergence (LP) application to V5-V6 soybeans; fb, followed by

[b]Values within a column that share a letter are similar according to Fisher's protected LSD ($\alpha = 0.05$)

[c]LP applications were not made within the 28 d after PRE application window and as such are not included in the corresponding analyses

Table 6.5 Effect of chloroacetamide herbicide on soybean injury 28 d after PRE application, and 28 d after LP application, averaged over application timing.[a,b]

Herbicide	Soybean injury 28 DAPRE	Soybean Injury 28 DALP
	----------(%)----------	
S-metolachlor	5a	2a
Acetochlor	2b	1b

[a]Abbreviations: DAPRE, days after preemergence (PRE) application immediately following planting; DALP, days after late postemergence (LP) application to V5-V6 soybeans; fb, followed by

[b]Values within a column that share a letter are similar according to Fisher's protected LSD ($\alpha = 0.05$)

Table 6.6 Effect of chloroacetamide herbicide by application timing interaction on weed density pooled over kochia, waterhemp, and Palmer amaranth data 28 d after PRE and soybean height at maturity.[a,b]

Chloroacetamide Herbicide	Application Timing	Density 28 DAPRE	Height at
		g m^{-2}	cm
S-metolachlor	PRE	10b	88ab
	PRE fb EP	28b	88ab
	PRE fb LP	42b	79fg
	EP	58b	83cde
	LP[c]	--	81efg
	EP fb LP	166a	79fg
Acetochlor	PRE	51a	86bc
	PRE fb EP	82a	82def
	PRE fb LP	64a	85bcd
	EP	64a	85bcd
	LP[c]	--	90a
	EP fb LP	64a	83de

[a]Abbreviations: DAPRE, days after preemergence (PRE) application immediately following planting; fb, followed by

[b]Values within a column that share a letter are similar according to Fisher's protected LSD ($\alpha = 0.05$)

[c]LP applications were not made within the 28 d after PRE application window and as such are not included in the corresponding analyses

Table 6.7 Effect of chloroacetamide application timing on weed density and biomass pooled over kochia, waterhemp, and Palmer amaranth data 28 d after LP; and soybean yield, averaged over chloroacetamide herbicide.[a,b]

Application Timing	Density 28 DALP	Biomass	Yield
	plants m^{-2}	g m^{-2}	kg ha^{-1}
PRE	17a	130a	2,017b
PRE fb EP	7b	9b	2,523a
PRE fb LP	8b	18b	2,560a
EP	8b	49b	2,545a
LP	5b	37b	2,367a
EP fb LP	13ab	41b	2,560a

[a]Abbreviations: DALP, days after late posteemergence (LP) application to V5-V6 soybeans; fb, followed by; PRE, preemergence application at planting; EP, early postemergence application to V2-V3 soybeans

[b]Values within a column that share a letter are similar according to Fisher's protected LSD ($\alpha = 0.05$)

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CHAPTER VII

OPTIMIZING CHLOROACETAMIDE APPLICATION TIMING IN DICAMBA-RESISTANT COTTON PRODUCTION SYSTEMS FOR CONTROL OF GLYPHOSATE-RESISTANT PALMER AMARANTH (*AMARANTHUS PALMERI* S. WATSON)

Abstract

In order to best steward the Xtend™ Weed Control System, best management practices such as mode of action diversification are necessary. Chloroacetamide herbicides effectively control small-seeded broadleaf and grass weeds and can be applied flexibly in cotton production systems. A chloroacetamide type x application timing factorial experiment was conducted in 2017 and 2018 in Mississippi to optimize chloroacetamide use in a dicamba-based Palmer amaranth management system for cotton production. Herbicides used were *s*-metolachlor and acetochlor, and application timings were PRE, PRE fb EP, PRE fb LP, EP alone, LP alone, and EP fb LP. Differences in cotton and weed response due to chloroacetamide type were minimal, and cotton injury following any application timing was under 10%. Late-season visible weed control was reduced by 20 to 56% if a single chloroacetamide application was used as opposed to a split application. Late-season weed densities were reduced 50 to 90% if split applications were used instead of single applications. Cotton height was reduced by up to 23% if a single application was made relative to split applications. Chloroacetamide applications at any timing except PRE alone minimized weed biomass at crop harvest. Yield was maximized by any split application timing whereas single applications resulted in a 20 to 60% yield loss. While no yield

loss was reported by delaying the first application (of a split application) until EP, foregoing a PRE application in hopes of timely POST applications is not advisable given the multiple factors that may delay timely POST applications such as inclement weather.

Nomenclature: Acetochlor; cotton, *Gossypium hirsutum* L.; dicamba; glyphosate; Palmer amaranth, *Amaranthus palmeri* S. Watson; *s*-metolachlor

Key words: Acetochlor, chloroacetamide, cotton, dicamba, *s*-metolachlor

Introduction

New commercially available cotton (*Gossypium hirsutum* L.) cultivars labeled for use in the Roundup Ready® XTEND Crop System (Bayer Corporation, Whippany, NJ 07981) contain engineered resistance to dicamba, glufosinate, and glyphosate (Behrens et al. 2007; Feng and Brinker 2014). The adoption of these cultivars has been widespread in the southern United States, with over 60% of cotton acres planted to the XTENDFLEX® (Bayer Corporation, 100 Bayer Boulevard, Whippany, NJ 07981) technology in 2017 (USDA 2017). High adoption rates of this technology likely reflect producers' desire to control herbicide-resistant (HR) weeds such as Palmer amaranth (*Amaranthus palmeri* S. Watson), utilize the best available crop germplasm, and as a defensive measure against off target dicamba deposition (Egan et al. 2014; Mortensen et al. 2012). The relatively low incidence of dicamba-resistant weed species renders this technology a promising tool for controlling a growing number of HR weed species (Heap 2019).

Palmer amaranth is one of the most problematic weeds in the U.S., and is the most deleterious weed in cotton production in the southern U.S. (Webster 2012, 2013). Palmer amaranth exhibits several characteristics that provide competitive advantages over cotton including rapid growth, large plant morphology, and prolific and extended reproduction

(Culpepper et al. 2010; Horak and Loughin 2000; Sellers et al. 2003; Webster and Grey 2015). Palmer amaranth populations as low as eight plants m^{-1} of row have been shown to reduce cotton yield by up to 92% (Morgan et al. 2001; Rowland et al. 1999). The development of Palmer amaranth with multiple resistance to glyphosate and acetolactate synthase (ALS) inhibitors has caused producers to shift to high utilization of herbicides with alternative modes-of-action (MOA), notably glufosinate and protoporphyrinogen oxidase (PPO) inhibitors (Sosnoskie and Culpepper 2014). This shift has already led to the development of Palmer amaranth populations with resistance to PPO inhibitors (Heap 2019; Salas et al. 2016). Multiple-resistant Palmer amaranth is a widespread problem in the southern United States, especially concerning populations with resistance to ALS inhibitors and glyphosate (Bagavathiannan and Norsworthy 2013). While resistance management strategies have been a focus in weed science for some time and have enjoyed some success, the continued development of herbicide resistant species is unlikely to abate due to producers adopting reactive strategies in lieu of proactive strategies (Meyer et al. 2015; Heap 2019). There is currently a relatively low incidence of dicamba-resistant weed species (Heap 2019), but Neve et al. (2011) demonstrated that 4,000 Palmer amaranth plants producing 250,000 seeds $plant^{-1}$ would be capable of producing five seeds with herbicide resistance if the mutation rate for resistance to a given herbicide is at least five per one billion individuals. Palmer amaranth and similar species with high fecundity and rapid seed production following germination are capable of reproducing in such numbers (Burke et al. 2007; Keeley et al. 1987; Massinga et al. 2001; Webster and Grey 2015). Furthermore, the genetic diversity present with such high reproductive rates is concerning regarding future development of resistance to herbicides such as dicamba, as research has shown that herbicide

resistance can arise due to subtle non-target-site mutations that are polygenic in nature as opposed to monogenic resistance which arises from target-site mutations (Delye et al. 2013; Jasieniuk et al. 1995; Jasieniuk et al. 1996; Vogwill et al. 2012).

In order to best steward dicamba-resistant weed control technology, the adoption of weed control strategies with a proactive focus on resistance prevention will be important moving forward. Previous research has shown the use of herbicides with multiple MOA tank-mixed and in rotation can help prevent the development of herbicide resistance (Beckie and Reboud 2009; Gressel and Segel 1990; Powles et al. 1997; Wrubel and Gressel 1994). Tank-mixing two herbicides can add up to four years before the onset of herbicide resistance development, as compared to rotating herbicides each year (Powles et al. 1997). Neve et al. (2003) simulated the development of HR in a 100 ha area with a beginning seed bank of 100 seeds m^2 and resistant gene frequency of one in 10^6 and found that resistance developing to each of two herbicides used as a tank-mix was unlikely within 50 years for all weeds. Conversely, when the herbicides were rotated in alternating years, multiple resistance to both herbicides arose in nearly all 100 ha areas (Neve et al. 2003).

Combinations of glyphosate and glufosinate with auxin herbicides such as 2,4-D or dicamba have been demonstrated to effectively control glyphosate-resistant (GR) Palmer amaranth at multiple stages of growth and densities (Cahoon et al. 2015b; Chahal and Johnson 2012; Merchant et al. 2013; Merchant et al. 2014; Vann et al. 2017). However, it is important to note that while glufosinate, glyphosate, and dicamba are labeled for use in XtendFlex™ cotton, much of the area utilizing this technology likely contains GR weeds. As such, in reality applications of combinations of dicamba, glufosinate, and glyphosate to such weeds contain two

effective herbicide MOA (glufosinate and dicamba) instead of three, and each of these herbicides are typically utilized POST. Research supports existing recommendations that call for the inclusion of residual herbicides along with POST herbicides in weed management plans focused on sustainably controlling Palmer amaranth (Culpepper et al. 2009; Everman et al. 2009; Gardner et al. 2006; Sosnoskie and Culpepper 2014). Chloroacetamide herbicides such as *s*-metolachlor and acetochlor are good candidates for tank mixing with dicamba due to their residual efficacy controlling weed emergence (thus reducing selection pressure on POST herbicides), crop safety, and application flexibility in terms of POST availability in a wide range of application rates. Braswell et al. (2016) found that fluridone or acetochlor can be used in a glufosinate-based Palmer amaranth control program to reduce use of/selection pressure on PPO-inhibiting herbicides.

Cahoon et al. (2015a) reported that the microencapsulated formulation of acetochlor (Warrant® Herbicide, Bayer Corporation) caused 3 to 8% less early-season injury to cotton than pendimethalin. Palmer amaranth control before POST application of glufosinate was 20% greater following a PRE application of acetochlor compared to pendimethalin, and was greater than control following PRE applications of diuron, fluometuron, or fomesafen (Cahoon et al. 2015a). Similarly, Geier et al. (2006) reported 80 to 100% control of Palmer amaranth by *s*-metolachlor and Steele et al. (2005) observed at least 91% control of Palmer amaranth following PRE application of *s*-metolachlor. However, crop safety is a concern in cotton production when considering the use of *s*-metolachlor as it has been shown to injure cotton following PRE use (Brown et al. 1993; Keeling et al. 1989), and is generally not recommended on sandy soils (Anonymous 2015) which account for many ha of cotton production in the southeast United

States. Stephenson et al. (2013) reported up to 7% cotton injury 3 d after treatment (DAT) following application of *s*-metolachlor alone to two- to three- lf cotton as compared to up to 31% injury if co-applied with pyrithiobac. *S*-metolachlor also slightly reduced cotton ht and node counts, although no yield reductions were reported from any treatment, implying only transient crop injury (Stephenson et al. 2013). Clewis et al. (2006) reported only minimal (3%) cotton injury following application of *s*-metolachlor alone or co-applied with either of two glyphosate formulations, and found the addition of *s*-metolachlor co-applied with glyphosate improved control of several weeds including Palmer amaranth by 6 to 46%. Everman et al. (2009) reported a yield increase of 200 kg ha^{-1} when *s*-metolachlor was included in POST applications of glufosinate compared to glufosinate applied alone, and a 13% decrease in Palmer amaranth control if residual herbicides were not included in PRE or mid-POST applications. Clewis et al. (2008) observed a 7 to 33% increase in annual broadleaf and grass control and a 420 kg ha^{-1} increase in cotton lint yield following co-application of *s*-metolachlor with glyphosate as opposed to glyphosate alone. Manuchehri et al. (2017) found co-application of acetochlor with glufosinate early postemergence (EPOST) in Enlist™ cotton (Corteva AgriSciences, Indianapolis, IN 46268) resulted in an 889 kg ha^{-1} increase in seedcotton yield relative to EPOST application of glufosinate alone. Cahoon et al. (2015a) reported 13 to 17% greater early-season Palmer amaranth control when acetochlor was co-applied with dicamba or fomesafen PRE. Tariq et al. (2018) reported reduced weed biomass 30 d after planting and increased cotton yield and economic returns following PRE application of *s*-metolachlor compared to pendimethalin, metolachlor, or combinations thereof.

Objective

In order to determine the optimal application timing and chloroacetamide herbicide for GR Palmer amaranth management in a dicamba-based cotton production system, research was conducted to evaluate weed control and crop response to two chloroacetamide herbicides applied at six different timings.

Materials and Method

Design and Treatment

Research was conducted at Dundee and Robinsonville, Mississippi in 2017 and 2018 to optimize the application timing of S-metolachlor and acetochlor for control of GR Palmer amaranth in dicamba-resistant cotton production. Four site-years of research were conducted, and site information is shown in Table 7.1. Each site contained a native GR Palmer amaranth population, which was present at a density of 100 to 150 plants m^{-2} at the Robinsonville location, and 65 to 91 plants m^{-2} at the Dundee location. The cotton cultivar 'DP1725B2XF' (DeltaPine®, Bayer Corporation, resistance to glyphosate, dicamba, and glufosinate) was seeded at 119,000 seeds ha^{-1} at a 2.5 cm depth. A two by six factorial arrangement of treatments arranged in a randomized complete block design with four replicates and a non-treated control (NTC) was utilized. Plots measured 12.2 m in length and consisted of four 97 cm rows, and were managed according to local production recommendations from the Mississippi State University Extension Service (Anonymous 2018). This research was conducted under dryland production systems as no irrigation was utilized.

Experimental factors were chloroacetamide herbicide (two levels) and herbicide application timing (six levels). The two chloroacetamide levels consisted of S-metolachlor (Dual

Magnum®, Syngenta US, Greensboro, NC 27419) and acetochlor (Warrant®, Bayer Corporation). The six application timing levels consisted of: (1) PRE immediately following planting, (2) PRE followed by (fb) early POST [PRE fb EP] at three to four lf cotton (Stewart et al. 2010), (3) PRE fb late POST [PRE fb LP] at pinhead square cotton (Stewart et al. 2010), (4) EP alone, (5) LP alone, and (6) split POST [EP fb LP]. A non-treated control (NTC) was included in the experimental design for comparison purposes. In order to compare similar concentrations of herbicide active ingredients, herbicide application rates were calculated based upon the maximum labeled application rates on the more restrictive of the two labels (*s*-metolachlor). As such, *s*-metolachlor applied PRE, EP, or LP alone was applied at 1.42 kg ai ha^{-1}, and acetochlor applied at these timings was applied at 1.27 kg ai ha^{-1}. The maximum amount of *s*-metolachlor labeled for POST use is 1.42 kg ai ha^{-1}, and as such a concentration of 0.71 kg ai ha^{-1} *s*-metolachlor was utilized for the split POST application treatment. Similarly, acetochlor was applied at a rate of 0.64 kg ai ha^{-1} per application for the split POST application treatment. The 12 treatment combinations utilized in this experiment are shown in Table 7.2 along with herbicide rate and cotton stage at application. In order to study these herbicides in the context of a dicamba-based weed control system, the maximum labeled rates of 0.56 kg ae ha^{-1} dicamba (XTENDIMAX®, Bayer Corporation) was included with all PRE applications, and 0.77 kg glyphosate ae ha^{-1} plus 0.39 kg dicamba ae ha^{-1} (Roundup XTEND™, Bayer Corporation) was included with all POST applications. Weed control programs in production systems utilizing the BOLLGARD II® XTENDFLEX® (Bayer Corporation) cotton cultivars also contain engineered resistance to glufosinate, which may be utilized for weed control in addition to glyphosate and dicamba. The focus of this research is to determine the most effective use of chloroacetamides in

a dicamba-based system. Therefore, only a single application of 0.66 kg ai ha^{-1} glufosinate (Liberty® 280 SL, BASF Corporation, Florham Park, NJ 07932) was made to all plots 28 d after LP following weed biomass collection to facilitate harvest, due to late-emerging Palmer amaranth's ability to disrupt mechanical harvesting equipment (Morgan et al. 2001; Smith et al. 2000). All herbicide applications were made using a spray boom with TTI11002 spray tips (TTI, TurboTee Induction, TeeJet Technologies, Glendale Heights, IL 60139) held 51 cm above the crop canopy. Herbicides were applied with a carbon dioxide-pressurized plot backpack sprayer operated at 4.8 KPH and calibrated to deliver 140 L ha^{-1} at 276 kPa. All herbicide applications were made to the center two rows of each four-row plot such that the outer two rows served as buffers between plots. Heat and precipitation accumulation varied by year, but annual totals fell within average historical ranges at each site year and are shown in Table 7.3. No irrigation was utilized at any site-year.

Data Collection and Analyses

Estimates of visible crop injury and Palmer amaranth control were made weekly from seven to 28 d after treatment (DAT) and ranged from zero (no visible injury) to 100 (complete plant mortality) (Frans et al. 1986). Visible cotton injury and weed control ratings were recorded at the listed interval relative to each application timing, such that the first rating period occurred 28 d after PRE application, and the last occurred 28 d after LP application. Palmer amaranth density was recorded by counting all live plants in a randomly selected 0.25 m^2 section of each plot 28 d after each application. Late-season Palmer amaranth biomass was harvested from this same 0.25 m^2 section of each plot following weed density collection 28 d after the LP application by cutting all plants in the 0.25 m^2 section at ground level and recording fresh weights. Fresh

biomass samples were then placed in a 40° C forced-air oven for 72 h and dry biomass was recorded. Cotton ht was recorded from the center two rows of each plot upon harvest aid application by randomly selecting six plants in each of the two center rows of each plot. Seedcotton yield was harvested using a two-row plot picker.

All data were analyzed with SAS 9.4 (SAS Institute, Inc., Cary, NC 27513). Studentized residuals were calculated for all data points, and those in excess of 2.5 were removed as outliers prior to ANOVA in order to avoid any outlier effects on analysis. Variance homoscedasticity was also evaluated and confirmed utilizing studentized residuals. Thus, all data met appropriate model and distribution assumptions and cotton injury, ht, and yield along with visible weed control, weed density and biomass data were each subjected to ANOVA using the SAS 9.4 PROC MIXED procedure, and means were separated using Fisher's protected LSD at the $\alpha = 0.05$ level of significance. A non-treated control (NTC) was included in the experiment for comparisons, but was not included in the analyses in order to minimize MSE and allow greater mean separation between response parameter means following each chloroacetamide application timing. The SLICE feature of the SAS 9.4 pdmix800 macro was utilized to conduct pairwise least squared means comparisons within PROC MIXED at the $\alpha = 0.05$ significance level (Saxton 1998). Year and location were combined as environment, which alone significantly affected cotton yield and Palmer amaranth control, but did not interact with either of the experimental factors (chloroacetamide, application timing) to affect response (data not shown). As such, environment may be considered a random effect given it only affected the magnitude of mean responses and not any trends. Chloroacetamide herbicide and application timing were analyzed as fixed effects and environment as a random effect. All data were pooled over

environment in order to estimate treatment effects over a wide variety of environments (Blouin et al. 2011; Carmer et al. 1989). This approach utilizing multiple-environment trials is useful for making broad inferences over time and space (Blouin et al. 2011; Carmer et al. 1989; Walker et al. 2008; Yang 2010) such as different soil types, precipitation, and heat accumulation displayed in Tables 7.1 and 7.3.

Results and Discussion

Evaluations 28 d After PRE

No two-factor interaction between chloroacetamide type and application timing was observed for any response parameter analyzed (data not shown). Both chloroacetamide type ($p = 0.0086$) and application timing ($p < 0.0001$) affected Palmer amaranth control 28 d after PRE (DAPRE), these effects are shown in Tables 7.4 and 7.5, respectively. Similarly, chloroacetamide type ($p = 0.0237$) and application timing ($p < 0.001$) each independently affected cotton injury evaluated 28 DAPRE (Tables 7.4 and 7.5). *S*-metolachlor provided 6% greater control of GR Palmer amaranth than acetochlor when evaluated 28 DAPRE and pooled over application timing (Table 7.4). The range in early season Palmer amaranth control evaluated 28 DAPRE (71 to 97% pooled across herbicides, 80 to 86% pooled across timings) is consistent with previous findings (Cahoon et al. 2015a; Geier et al. 2006; Steele et al. 2005). However, this increase in activity was also reflected in terms of cotton injury 28 DAPRE, which was 2% greater following application of *s*-metolachlor PRE than acetochlor when pooled over application timing (Table 7.4), similar to previous findings of increased injury potential on cotton treated with *s*-metolachlor (Brown et al. 1993; Keeling et al. 1989). While injury was noted, it did not exceed 10% following any application timing or from either herbicide, although

s-metolachlor did cause slightly more injury than acetochlor. Similar cotton injury following chloroacetamide use has been noted in previous research (Clewis et al. 2006; Stephenson et al. 2013). Palmer amaranth control 28 DAPRE was maximized by chloroacetamide application PRE fb EP, and was reduced by 24 to 26% if initial application was delayed until EP (Table 7.5). Conversely, cotton injury was 6 to 8% greater 28 DAPRE following chloroacetamide application PRE fb EP compared to PRE alone or PRE fb LP (Table 7.5). Palmer amaranth density 28 DAPRE was affected by application timing ($p < 0.0001$). Delaying application until EP resulted in a 50 to 65% increase in Palmer amaranth density compared to initial application occurring PRE when pooled over chloroacetamide type (Table 7.6). Application of a chloroacetamide PRE fb EP minimized Palmer amaranth density relative to all other timings (Table 7.6).

Evaluations 28 d After EP

No two-factor interaction between chloroacetamide type and application timing was observed for any response parameter analyzed (data not shown). At 28 d after EP (DAEP) Palmer amaranth control and density and cotton injury were affected by application timing ($p < 0.0001$ each). Palmer amaranth control at 28 DAEP ranged from 48 to 94% (Table 7.5). Maximum visible control (90 to 94%) was observed following chloroacetamide application PRE fb EP, PRE fb LP, or EP fb LP (Table 7.5) and was similar to control reported in previous research (Cahoon et al. 2015a; Geier et al. 2006; Steele et al. 2005). Similarly, maximum cotton injury 28 DAEP was observed following chloroacetamide application PRE fb EP or EP fb LP, although the maximum injury observed was 3% (Table 7.5). Palmer amaranth density was minimized by chloroacetamide application at PRE fb EP, PRE fb LP, or EP fb LP (Table 7.6). At this

evaluation timing, the PRE application provided greater density reduction than if the initial application was delayed until LP (9 vs 13 plants m^{-2}, respectively, Table 7.6).

Late Season Evaluations

No two-factor interaction between chloroacetamide type and application timing was observed for any response parameter analyzed (data not shown). Palmer amaranth control, density, and cotton injury 28 d after LP (DALP) were affected by chloroacetamide application timing ($p < 0.0001$ each). Maximum Palmer amaranth control 28 DALP was achieved by any split chloroacetamide application: PRE fb EP, PRE fb LP or EP fb LP (Table 7.5), consistent with Cahoon et al. (2015b) who reported improved control of Palmer amaranth following multiple applications of dicamba (each POST application in this experiment included dicamba plus glyphosate). Single POST applications resulted in 69 to 76% control and a PRE only application provided only 48% control (Table 7.5), which is similar to Everman et al. (2009) which reported improved cotton yield following POST co-application of *s*-metolachlor with glufosinate compared to glufosinate alone. Cotton injury 28 DALP was minor (5% or less), with maximum injury occurring following a single chloroacetamide application LP (Table 7.5). This low magnitude of cotton injury corresponds with previous findings that cotton injury following chloroacetamide application is minor and transient (Clewis et al. 2006; Stephenson et al. 2013). Palmer amaranth density 28 DALP was minimized by all split application timings: PRE fb EP, PRE fb LP, or EP fb LP (Table 7.6). While it is intuitive that POST applications of dicamba plus glyphosate would lead to improved control of emerged Palmer amaranth, dicamba and glyphosate have little to no residual activity and therefore any differences in late season Palmer amaranth densities (28 DALP) can reasonably be attributed to chloroacetamide activity. Palmer

amaranth dry biomass recorded at cotton defoliation was affected by chloroacetamide application timing ($p < 0.0001$). Biomass ranged from 4 to 50 g m^{-2}, and was minimized by any application timing other than PRE alone (Table 7.6). While late-season weed biomass was similar following all application timings except PRE alone, the prolonged weed-crop competition enabled by delaying initial applications until POST may manifest more severely in areas with more severe infestations or when producers cannot make timely POST applications. Similarly, while all other application timings resulted in similar densities, split applications (PRE fb LP and EP fb LP) trended numerically less than those that included only a single application (EP or LP alone, Table 7.6), and these numeric trends may become more pronounced with heavier Palmer amaranth infestations. Cotton ht at maturity and seedcotton yield were each affected by chloroacetamide application timing ($p < 0.0001$). Cotton ht was maximized following chloroacetamide application PRE fb EP (80cm, Table 7.6) and reduced by 10 to 23% if initial application was delayed until EP or LP, respectively (Table 7.6). This height reduction likely reflects the deleterious effect of allowing weed-crop competition to continue unchecked until POST, even in the case of a split-post application (EP fb LP). It is worth considering that some height reduction at maturity may also be due to cotton injury following late-season chloroacetamide applications since Stephenson et al. (2013) reported reduced cotton ht following exposure to *s*-metolachlor. However, the low magnitude of cotton injury observed 28 DALP (≤5%) seemingly supports the height reduction being due to weed-crop competition and not herbicide injury. Similarly, if a PRE application was made, delaying the second application until LP led to an 8% reduction in cotton ht at maturity relative to the second application occurring EP. Cotton yield was maximized by all split application timings: PRE fb EP, PRE fb LP, or EP

fb LP (Table 7.6), consistent with previous research that found 200 to 889 kg ha^{-1} increases in cotton yield following co-application of chloroacetamide herbicides with glyphosate or glufosinate POST as opposed to the POST herbicides alone (Clewis et al. 2008; Everman et al. 2009; Manuchehri et al. 2017; Tariq et al. 2018). It is important to note that plots that received the split POST application timing (EP fb LP) received half of the total chloroacetamide rate of the application timings involving a split PRE and POST application (Table 7.2). Conversely, if this same concentration is applied in a single POST application with no PRE (EP and LP alone), yield was reduced by up to 743 kg ha^{-1}, or 25% (Table 7.6). This may imply that utilizing multiple chloroacetamide applications leads to improved yield over single applications, even if all applications are made POST and the same total volume of herbicide delivered is equivalent.

Significance of Findings for Cotton Management

In order to best steward new dicamba-based weed control technologies, best management practices regarding herbicide use and resistance management should be observed. One component of proper resistance management and herbicide stewardship is the use of multiple herbicide MOA in a weed control plan. This research sought to optimize the use of one part (chloroacetamides) of a more comprehensive dicamba-based weed control plan. Acetochlor and *s*-metolachlor each provide good residual control of GR Palmer amaranth. In order to reduce weed-crop competition and selection pressure on POST herbicides such as dicamba or glufosinate, a split application of either *s*-metolachlor or acetochlor is advisable. Foregoing a PRE application of either herbicide in favor of a split POST application may result in similar yields as applications that include both a PRE and POST application, but doing so may introduce the possibility for reduced cotton height, which may translate to yield loss in more severe

scenarios. Similarly, delaying initial application until POST may lead to increased weed biomass at harvest, which can impede mechanical cotton harvest (Morgan et al. 2001; Smith et al. 2000). Furthermore, while similar yields may result regardless of the first of a split chloroacetamide application occurring PRE vs. POST, delaying initial application until POST puts additional selection pressure on POST herbicides such as dicamba and glyphosate due to increased early- and mid-season weed densities. A split application of either *s*-metolachlor or acetochlor PRE fb EP or LP is recommended as one part of a comprehensive dicamba-based GR Palmer amaranth management program, though multiple POST applications offer effective recourse if a timely PRE application cannot be made.

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Tables

Table 7.1 Location, year, longitude, latitude, elevation, soil type, and planting, application, and harvest dates for each experiment site-year.[a]

Location	Yea	Longitud	Latitud	Elevatio	Soil	Application Date			Harve
				m		PRE	EP[d]	LP[e]	
Dundee	201	90° 28’	34° 32’	40	Sharkey	May	Jun	Jun	October
Dundee	201	90° 28’	34° 32’	40	Sharkey	May	Jun	Jun	October
Robinsonvil	201	90° 29’	34° 83’	60	Commer	May	Ma	Jun	October
Robinsonvil	201	90° 29’	34° 83’	60	Commer	May	Ma	Jun	October

[a]Abbreviations: PRE, preemergence; EP, early postemergence; LP, late postemergence
[b]Source: U.S. Department of Agriculture, Natural Resources Conservation Service (2019) http://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx
[c]PRE applications occurred immediately after planting on the same day
[d]EP applications were made to cotton at the 3 to four leaf growth stage
[e]LP applications were made to cotton at the pinhead square growth stage

Table 7.2 Concentrations of acetochlor or *s*-metolachlor applied PRE, EP, or LP immediately after planting, to three to four lf cotton, or to pinhead square cotton, respectively.[a]

Herbicide[b]	Treatment Combination	Application Timing		
		PRE[c]	EP[d,e]	LP[d,f]
s-metolachlor (Syngenta)	1	1.42 kg ai ha^{-1}	--	--
	2	1.42 kg ai ha^{-1}	1.42 kg ai ha^{-1}	--
	3	1.42 kg ai ha^{-1}	--	1.42 kg ai ha^{-1}
	4	--	1.42 kg ai ha^{-1}	--
	5	--	--	1.42 kg ai ha^{-1}
	6	--	0.71 kg ai ha^{-1}	0.71 kg ai ha^{-1}
Acetochlor (Bayer CropScience)	7	1.27 kg ai ha^{-1}	--	--
	8	1.27 kg ai ha^{-1}	1.27 kg ai ha^{-1}	--
	9	1.27 kg ai ha^{-1}	--	1.27 kg ai ha^{-1}
	10	--	1.27 kg ai ha^{-1}	--
	11	--	--	1.27 kg ai ha^{-1}
	12	--	0.64 kg ai ha^{-1}	0.64 kg ai ha^{-1}

[a]Abbreviations: PRE, preemergence; EP, early postemergence; LP, late postemergence
[b]An application of 0.66 kg ai ha^{-1} glufosinate (Liberty® 280 SL, BASF) was made to all plots 28 d after LP to facilitate harvest
[c]All PRE applications included 0.56 kg ae ha^{-1} dicamba (XTENDIMAX®, Bayer Corporation) made immediately following planting
[d]All POST applications included 1.68 kg ae ha^{-1} dicamba plus glyphosate (Roundup XTEND™, Bayer Corporation)
[e]EP applications were made to cotton at the 3 to four leaf growth stage
[f]LP applications were made to cotton at the pinhead square growth stage

Table 7.3 Native weed density and precipitation and heat accumulation totals 14 days after each application and season-long at each site year.[a]

Location	Year	Palmer amaranth density	14 DA-PRE[b]		14 DA-EP[c]		14 DA-LP[d]		Total[e]	
			Rainfall	DD60	Rainfall	DD60	Rainfall	DD60	Rainfall	DD60
		plants m^{-2}	cm		cm		cm		cm	
Dundee	2017	65-91	7.1	171	5.5	246	1.9	286	36.0	2696
Dundee	2018	65-91	0.5	285	0.4	305	0.3	250	31.4	2883
Robinsonville	2017	100-150	3.3	183	8.0	210	2.6	267	35.5	2391
Robinsonville	2018	100-150	0.6	250	1.9	290	0.1	311	26.4	2772

[a]Abbreviations: 14 DA-PRE, fourteen days after preemergence application made immediately following planting; 14 DA-EP, fourteen days after early postemergence application to cotton at the 3 to 4 lf growth stage; 14 DA-LP, fourteen days after late postemergence application to cotton at the pinhead square growth stage; DD60, growing degree days (calculated by subtracting 60 from the average daily temperature)
[b]Precipitation and DD60 data in this section are cumulative totals from the 14 days immediately following PRE herbicide application
[c]Precipitation and DD60 data in this section are cumulative totals from the 14 days immediately following EP herbicide application
[d]Precipitation and DD60 data in this section are cumulative totals from the 14 days immediately following LP herbicide application
[e]Precipitation and DD60 data in this section are cumulative totals from the entire growing season between the PRE and harvest dates shown in Table 7.1

Table 7.4 Effect of *s*-metolachlor or acetochlor on Palmer amaranth control and density and cotton injury 28 d after PRE application, averaged over application timing.[a,b]

Herbicide	Palmer amaranth Control	Cotton Injury
	----------%----------	
S-metolachlor	86a	8a
Acetochlor	80b	6b

[a]Abbreviations: PRE, preemergence
[b]Values within a column that share a letter are similar according to Fisher's protected LSD (α = 0.05)

Table 7.5 Effect of chloroacetamide application timing on Palmer amaranth control and cotton injury 28 d after PRE, EP, and LP, averaged over chloroacetamide herbicide.[a,b]

Application Timing	Control 28 DAPRE	Control 28 DAEP	Control 28 DALP	Injury 28 DAPRE	Injury 28 DAEP	Injury 28 DALP
	----------%----------					
PRE	86b	48d	40c	2c	1c	0c
PRE fb EP	97a	93a	96a	10a	1c	1bc
PRE fb LP	87b	90ab	93a	4c	3ab	1bc
EP	71c	83b	76b	8b	1c	0c
LP[c]	--	69c	69b	--	2bc	5a
EP fb LP	73c	94a	95a	9ab	4a	2b

[a]Abbreviations: DAPRE, days after preemergence (PRE) application immediately following planting; DAEP, days after early postemergence (EP) application to cotton at the 3 to 4 lf growth stage; DALP, days after late posteemergence (LP) application to pinhead square cotton; fb, followed by

[b]Values within a column that share a letter are similar according to Fisher's protected LSD (α = 0.05)

[c]LP applications were not made within the 28 d after PRE application window and as such are not included in the corresponding analyses

Table 7.6 Effect of chloroacetamide application timing on Palmer amaranth density 28 d after PRE, EP, and LP; Palmer amaranth biomass and cotton ht at cotton defoliation; and seedcotton yield, averaged over chloroacetamide herbicide.[a,b]

Application Timing	Density 28 DAPRE	Density 28 DAEP	Density 28 DALP	Biomass	Height	Yield
	----------plants m^{-2}----------			g m^{-2}	cm	kg ha^{-1}
PRE	7c	9b	10a	50a	71c	1,219c
PRE fb EP	2d	3d	3c	14b	80a	2,824a
PRE fb LP	8bc	4cd	3c	8b	74b	2,958a
EP	13a	6c	6b	17b	71c	2,352b
LP[c]	--	13a	9a	15b	62d	2,215b
EP fb LP	12ab	3d	1c	4b	72bc	2,927a

[a]Abbreviations: DAPRE, days after preemergence (PRE) application immediately following planting; DAEP, days after early postemergence (EP) application to cotton at the 3 to 4 lf growth stage; DALP, days after late posteemergence (LP) application to pinhead square cotton; fb, followed by

[b]Values within a column that share a letter are similar according to Fisher's protected LSD (α = 0.05)

[c]LP applications were not made within the 28 d after PRE application window and as such are not included in the corresponding analyses

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APPENDIX A

A BRIEF HISTORY OF WEED CONTROL AND ITS STATUS IN COTTON PRODUCTION

Abstract

Cotton has been one of the most widely-cultivated crops in history. Market conditions in recent history led to a decrease in cotton production domestically and in the Mid-South, but improved current market conditions and the launch of new weed control technologies have cotton production poised to increase in the near future. Cotton producers both new and old must be cognizant of the lessons from past challenges in weed control including weed control method development and the onset of herbicide resistance in order to steward these new technologies. An understanding of cotton history and weed control in the past and present may aid producers and the cotton community in shaping an informed, responsible approach to production in the immediate future. Here we present a brief history on the discovery, domestication, and production of cotton in the Mid-South as well as a brief history on the formation and development of weed science and weed control practices. Topics include the development of herbicide-resistant weeds, glyphosate resistant crops and weeds, 2,4-D and dicamba mode-of action and selectivity, the risks associated with improper use of 2,4-D- and dicamba- resistant cultivars, susceptible cotton response to 2,4-D and dicamba, and the future outlook on 2,4-D and dicamba use in cotton production. Ultimately, we hope to provide the background information necessary for new cotton producers to understand cotton production in the context of weed control history so that they might be better stewards of new weed control technologies.

Nomenclature: 2,4-D, 2,4-dichlorophenoxyacetic acid; corn, *Zea mays* L. ZEAMX; cotton, *Gossypium hirsutum* L. GOSHI; dicamba, 3,6-dichloro-2-methoxybenzoic acid; glyphosate, N-(phosphonomethyl)glycine; soybean, *Glycine max* (L.) Merr. GLXMA.

Key words: 2,4-D, auxin, cotton, dicamba, history, resistance

History of Cotton

Upland cotton (*Gossypium hirsutum* L.) has been one of the most widely-cultivated crops in history, playing an important economic role in fiber/textile, seed oil, and protein meal production (Brubaker et al. 1999). Seelanan et al. (1997) elucidated DNA sequencing data that suggests an ancestral progenitor may have arisen in the *Gossypium* genus some 10 to 20 million years ago. Following the arrival of this original *Gossypium* forebear, radiative evolution gradually led to the development of four extant cotton species: *G. arboretum*, *G. barbadense*, *G. herbaceum*, and *G. hirsutum* (Brubaker et al. 1999). Unlike many other crops, cotton domestication occurred independently in four distinct instances, with *G. arboretum* and *G. herbaceum* arising in the Old World and *G. barbadense* and *G. hirsutum* arising in the New World (Brubaker et al. 1999) *G. hirsutum*, which accounts for over 90% of annual cotton production across the globe, was originally domesticated in the Yucatan Peninsula of ancient Mesoamerica but now is cultivated as far north as the central United States to as far south as Australia and lower South America (Brubaker and Wendel 1994; Niles and Feaster 1984). Archaeobotanical remains from the Tehaucan Valley in Mexico suggest that cotton domestication had occurred by at least 4,000 to 5,000 years ago (Brubaker and Wendel 1994). Originally exhibiting a growth habit akin to a perennial shrub or sapling with small, sparsely-haired seeds; domestication and cultivar improvement has led to development of the compact, bushy annual cotton plants with large seeds and long white fibers grown today (Brubaker et al.

1999). Interestingly, the second layer of shorter, coarser lint fibers colloquially known as linters do not appear to serve an evolutionary purpose, indicating that they are likely the product of early domestication selecting towards some unknown end use (Hutchinson et al. 1947).

Cotton Production in the Mid-South

As poor lint prices persist and a growing number of synthetic fibers and other manufactured products provide alternatives to cotton lint, world production has been on a decline for the last several years. Cotton consumption, however, has remained steady and is in fact projected to increase in 2016/2017 (Johnson et al. 2016) Cotton production in Mississippi and the rest of the Mid-South has reflected these national production trends and declined steadily since the late 1990's and early 2000's. However, production in Mississippi in 2016 was the highest it had been in four years at 182,000 hectares planted (USDSA-NASS 2016). Furthermore, low prices and government support, poor weather, and high pest pressure will ostensibly lead to a loss of nearly 11 million bales of cotton produced in China, India, and Pakistan in 2016/2017. Coupled with the relatively steady global lint consumption, these conditions could likely lead to an increased lint price and thus more production in coming years (Johnson et al. 2016).

Development of Herbicide-Resistant Weeds

Humans have been cultivating crops for anywhere between 10,000 to 15,000 years, and have been practicing pest control methods since as early as 3000 B.C. (Timmons 1970). Weed management is an integral component of any crop production system due to weed species' ability to severely reduce crop yields by competing for resources such as light, water, and nutrients (Mithila et al. 2011; Mortensen et al. 2012). The frustrating reality producers face is that weeds are virtually impossible to completely remove from a given field, and thus weed control will always be a component of crop production (Mortensen et al. 2012). The dawn of

modern herbicides in weed control began in the early 1940's. Shortly after the beginning of commercial herbicide development, a cognizance of the potential for the development of herbicide-resistant (HR) weed species began to emerge in 1950 (Appleby 2005). Weed scientists in the early 1950's began to notice the declining efficacy of some types of herbicides on previously controlled weed populations, and could distinguish previously inconspicuous differences between weed biotypes of the same species based solely on susceptibility to herbicides (Harper 1956). After its commercialization in 1947, 2,4-D was widely used due to its effective and selective control of broadleaf weed species, particularly in monocot or pasture crops. However, owing to 2,4-D's selectivity for broadleaf weeds, additional inputs were necessary for controlling grassy weeds. As 2,4-D use continued to increase, a weed shift towards monocot weed dominance followed and was reflected in the corresponding increase in research on common grass weeds such as large crabgrass (*Digitaria sanguinalis* L.) and quackgrass (*Elymus repens* L.) (Mithila et al. 2011). In 1962, Hanson reported differences in 2,4-D sensitivity among spreading dayflower (*Commelina diffusa*) biotypes in Hawaii, an area with heavy 2,4-D usage in sugarcane production.

The triazine herbicides followed the commercialization of 2,4-D and other synthetic auxins, and were introduced in the 1950's (Heap 2017). Heavy use of this new chemistry led to the accelerated development of weeds with triazine-resistance, of which there are currently 74 documented species (Heap 2017). Triazine herbicide use led to another shift in the weed community to the predominance of species with late growing season germination such as redroot pigweed (*Amaranthus retroflexus* L.) and fall panicum (*Panicum dichotomiflorum*) (Mithila et al. 2011; Triplett and Lytle 1972). Next came the dawn of the ALS-inhibiting herbicides in the 1980's. These herbicides were immediately and widely used in production of

the major row crops, and once again resistant weed biotypes developed, and ultimately culminated in the 159 species with confirmed resistance today (Heap 2017; Mithila et al. 2011). Further exacerbating the issue of herbicide resistance has been the development of weed biotypes with resistance to herbicides of different modes-of-action, also referred to as having multiple-resistance. Initial suppositions held that the development of multiple-resistance would only occur on an exceedingly rare basis. Substantiating this ideology was the belief that multiple resistance could only occur from two or more discrete mutation events (each with a frequency on the order of one resistant individual for every 10^5 to 10^{10} individuals) arising in the same population and thus conferring multiple resistance (Délye et al. 2013). However, the recent development of nearly 40 weed species with resistance to two or more herbicide modes of action testifies to mother nature's ability to defy the odds (Beckie and Tardif 2012; Délye et al. 2013; Mortensen et al. 2012). Such rapid development and spread of weed species with multiple-resistance implies that multiple-resistance is likely not the product of extremely infrequent mutation events, but rather it occurs when a different herbicide mode of action is overused to control a weed population that has already developed resistance to one mode of action through previous herbicide use (Mortensen et al. 2012).

Weed species that have obtained a single mechanism allowing for the rapid metabolism of herbicides from different chemical families through a process called cross-resistance complicate the issue of herbicide resistance even further. Cross-resistance can be conferred commonly through mutations to the cytochrome P450 monooxygenase genes since most plant species contain many such genes, which encode proteins utilized in metabolism/detoxification of foreign molecules (Mortensen et al. 2012; Powles and Yu 2010). Similarly, some species have developed multiple mechanisms conferring resistance to herbicides from different chemical

families and modes of action through a process called multiple resistance (Heap 2017; Mortensen et al. 2012; Powles and Yu 2010). The development of multiple and cross-resistance in weed species is especially troubling as it compounds the danger to herbicides' utility as weed control tools.

Glyphosate Resistant Crops and Weeds

The most recent case of widespread herbicide resistance in weeds began in 1996 with the development of genetically modified (GM) transgenic crops with resistance to glyphosate (Roundup Ready® crops from Monsanto Company). This development led to great advances in simplifying and improving weed control (Duke and Powles 2009; Mortensen et al. 2012). The process of weed control in crop production transformed from the careful selection of herbicides to the more streamlined use of glyphosate in tandem with GM crops, owing to glyphosate's high efficacy as a broad-spectrum herbicide adept at controlling a great number of weed species (Duke and Powles 2009; Mortensen et al. 2012). However, these advances have also led to an overreliance on glyphosate, with 54% of all soybean acres utilizing GR cultivars by 2000, and 92%, 63%, and 68% of all US soybean, corn, and cotton hectares, respectively, being planted to GR cultivars by 2008 (Mortensen et al. 2012). Overall, by 2008 GR crops were planted on 96 million hectares of land internationally, a trend that greatly contributed to the overuse of glyphosate, ultimately culminating in the development and endemic spread of glyphosate-resistant (GR) weed species (Duke and Powles 2009). This occurrence was originally surprising as the prevailing opinion was that the development of GR weed species was unlikely owing to how complex the process of engineering GR crops was (Bradshaw et al. 1997). Nonetheless, the rapid and nearly ubiquitous use of glyphosate and GM crops with resistance to glyphosate coupled with reduced tillage systems resulted in a series of subsequent weed shifts and

resistance development (Culpepper 2006; Mithila et al. 2011). In 2006, Firbank et al. showed that GR corn fields exhibited higher weed seedbank density than fields utilizing a conventional weed control system. In Indiana, late-season weed escapes in 2003, 2004, and 2005 were most commonly composed of GR horseweed (*Conyza canadensis),* GR giant ragweed (*Ambrosia trifida*), and GR common lambsquarters (*Chenopodium album*); all large-seeded summer annual weeds that began to dominate weed communities upon the introduction of reduced tillage in tandem with GR technology (Davis et al. 2008). Jeschke and Stoltenberg (2006) demonstrated that after eight years of continued glyphosate use in a corn-soybean rotation, weed species composition shifted from a diverse community of common lambsquarters, pigweed (*Amaranthus*) species, and giant foxtail (*Setaria faberi*) to one primarily composed of giant ragweed, large crabgrass (*Digitaria sanguinalis*), and shattercane (*Sorghum bicolor* L.). Furthermore, a two-year study characterizing weed species shifts in GR soybean systems showed that continued use of glyphosate resulted in the ability of ivyleaf morningglory (*Ipomoea hederacea*) and shattercane to bolster the seedbank at rates higher than other weeds (Hilgenfield et al. 2004).

In 1996, rigid ryegrass (*Lolium rigidum*) became the first weed with confirmed glyphosate resistance; the first of some 36 species with currently documented resistance (Heap 2017). However, horseweed was the first GR weed that arose from a production system with GM crops containing the GR trait, and the third GR weed identified overall (Mithila et al. 2011; VanGessel 2001). The continued development and spread of HR weed species has become an issue of primary concern to crop producers globally. In the Mid-South region of the United States, species such as palmer amaranth (*Amaranthus palmeri*), horseweed, and Johnsongrass (*Sorghum halepense*) pose a significant challenge to producers utilizing glyphosate-resistant

cultivars (Heap 2017; Mortensen et al. 2012). Since the advent of GR crop technology in 1996, weed populations have shifted to highly troublesome weeds such as giant ragweed, horseweed, lambsquarters, morningglory, pigweed, and shattercane (Mithila et al. 2011).

Auxin Herbicide Mode of Action and Symptomology

In response to this growing threat of herbicide-resistant weeds private industry companies have recently developed new GM crop technologies with genetic traits conferring resistance to the synthetic auxins 2,4-D (Dow AgroScience's Enlist®) or dicamba (Monsanto Company's XTEND® Cropping System). These technologies are built around these novel GM cultivars and the use of new formulations of 2,4-D and dicamba, which will each be used in tandem with glyphosate in these new weed control systems. While these GM biotechnologies are novel, the herbicides they are based around are quite old. Originally synthesized in 1941, 2,4-D was one of the first synthetic herbicides used in commercial agriculture after its utility as a herbicide was reported in 1942 (Pokorny 1941; Zimmerman and Hitchcock 1942). Dicamba was discovered and patented 16 years later by S.B. Richter in 1958 (Senseman 2007). Due to their herbicidal effects on plant hormone pathways and growth and development via disruption of the auxin-hormone pathway, 2,4-D, dicamba, and similar molecules such as quinclorac and clopyralid are classified as synthetic auxins (Senseman 2007). Despite the fact that these herbicides have been used for nearly seven decades, their specific mode of action is still poorly understood. Currently their mode-of-action (MOA) is categorized as synthetic auxins, which fit under the broader classification of plant growth regulator (PGR) herbicides. The herbicidal activity of these synthetic auxins is described as affecting cell wall plasticity, nucleic acid metabolism, cell elongation, and vascular tissue disruption (Senseman 2007). Recent advances have identified putative auxin-response pathways at low and high auxin concentration (Figure A.1),

characterized by Song in 2014. At low levels of the native endogenous auxin hormone indole-3-acetic acid (IAA), Aux/IAA active repressor proteins bind auxin-response factor (ARF), effectively inhibiting synthesis of auxin-response genes (Mockaitis and Estelle 2008). At high levels of IAA or its exogenous auxin herbicide analogs, influx channels allow the IAA/analog to enter the cell, bind the Aux/IAA repressors, and catalyze their degradation with mediation from the F-box protein TIR_1. Thus, ARF is liberated and free to promote auxin-response gene synthesis (Tan et al. 2007). Auxin-response gene synthesis results in a cocktail of familiar molecules being synthesized, including ethylene, abscisic acid, reactive oxygen species and nitrous oxide. Each of these molecules contributes to the characteristic epinasty and eventual plant death caused by application of synthetic auxin hormones (Song 2014).

Once a susceptible plant has been exposed to 2,4-D or dicamba, very conspicuous and characteristic symptomology follows suit. Common early visual symptoms include the bending and twisting of stem and petiole tissue often called epinasty, stem swelling at the nodes, the formation of callus tissue, internode elongation, leaf cupping, strapping, curling, and malformation, and abnormal venation (Byrd et al. 2015; Marple et al. 2007; Senseman 2007). Eventually, growing points exhibit chlorosis, halted growth, wilting, and eventual necrosis. The duration of these symptoms and eventual plant death occurs slowly and can take up to five weeks (Marple et al. 2007; Senseman 2007). If exposure to a low concentration of auxin herbicide occurs, nascent leaf tissue may pucker and develop narrow extensions of leaf midribs instead of leaf tips. (Senseman 2007). Various other symptoms may be caused by exposure to the auxin herbicides, and symptomology depends on a multitude of factors including species, herbicide molecule, herbicide rate, and growth stage at the time of exposure.

2,4-D and Dicamba Characteristics and Selectivity

2,4-D and dicamba are among the most commonly used synthetic auxin herbicides in modern crop production and as a whole account for up to 10% of all herbicides used (Burnside 1996), and potentially more since the development of widespread herbicide-resistance. In data from 2013 rice (*Oryza sativa*) and peanut (*Arachis hypogaea*) production, 2014 corn (*Zea mays*) production, and 2015 cotton, wheat (*Triticum* spp.), soybean (*Glycine max*) and oat (*Avena sativa*) production systems alone, nearly 19 million pounds of 2,4-D and almost 3 million pounds of dicamba herbicides were used (Table A.1) (USDA-NASS 2017). Such high use of these herbicides testifies to their lasting utility in weed control after being developed decades ago following World War II, their low cost relative to newer herbicides, and their minimal health risks except to aquatic plant and amphibian species (Mississippi Cooperative Extension Service 1997; Sciumbato et al. 2004a; USEPA 2005, 2006). The two herbicides share structural similarity both with each other and with IAA (Figure A.2) and behave similarly in their control of weeds.

2,4-D

Classified as a weak acid (pK_a = 2.8), 2,4-D is used as a foliar-applied herbicide for postemergence (POST) control of broadleaf weeds in turf, pasture, fallow, and Conservation Reserve Program (CRP) systems; and in cereal grains such as wheat, oats, and sorghum (*Sorghum bicolor*) at rates ranging from 0.28 to 0.56 kg ae ha^{-1} in cereals and up to 2.24 kg ae ha^{-1} in turf, pasture, fallow, and CRP systems (Senseman 2007). 2,4-D is also commonly used as a preplant (PRPL) or POST option for weed control in field corn up to 0.56 kg ae ha^{-1}, PREPL in soybeans up to 1.12 kg ae ha^{-1}, preharvest (PREHV) in wheat up to 1.7 kg ae ha^{-1}, and even in aquatic weed control at rates up to 22.4 kg ae ha^{-1}. Additionally, 2,4-D may be used for selective

weed control in some fruit and vegetable production systems, including asparagus (*Asparagus officinalis*), dormant strawberries (*Fragaria* spp.), and fruit trees such as apples (*Malus pumila*), peaches (*Prunus persica*), and pears (*Pyrus* spp.) (Senseman 2007).

Primarily used as a broadleaf herbicide for the control of dicotyledonous weed species, 2,4-D is especially effective in selectively controlling troublesome weed species such as pigweed (*Amaranthus* spp.) species, horseweed, giant ragweed (*Ambrosia trifida*), velvetleaf (*Abutilon theophrasti*), and common lambsquarters. 2,4-D does not affect most monocotyledonous (grass) species, making it a particularly attractive option for use in pasture or cereal production systems. Upon application, plant roots and foliage each absorb 2,4-D herbicide provided there is a rain-free period of at least four hours. Once absorbed, 2,4-D is mobile and translocates symplastically, entering meristematic tissue cells of the plant's growing points in both root and shoot tissue via both passive diffusion and active transport. After reaching meristem tissue, it is metabolized very slowly and thus has prolonged herbicidal activity (Senseman 2007, Wall et. al 1991). 2,4-D is not particularly burdensome on the environment since it is rapidly metabolized by soil-dwelling microbes, as demonstrated by its field half-life of 10 days, average soil movement of under 15 centimeters, and minimal leaching (Senseman 2007). Toxicologically, 2,4-D is relatively safe for use given its high oral and dermal LD_{50} values and minimal reported effects on acute or chronic toxicity as well as mutagenicity, reproduction, or chronic exposure (Senseman 2007).

Dicamba

Like 2,4-D, dicamba is classified as a weak acid and is primarily used as a POST herbicide, though it may be used PREPL at 0.56 kg ae ha^{-1} in corn and sorghum. Common use rates for selective POST control of broadleaf weeds are 0.28 kg ae ha^{-1} in corn and sorghum, up to 0.14

kg ae ha^{-1} in small grains, up to 2.24 kg ae ha^{-1} in pasture, up to 2.2 kg ae ha^{-1} in fallow, and up to 1.1 kg ae ha^{-1} in turf. Additionally, dicamba may be used at rates up to 0.56 kg ae ha^{-1} in asparagus production in California, Washington, and Oregon (Senseman 2007). Also like 2,4-D, dicamba is commonly used for selective control of annual broadleaf weed species such as pigweed species, common lambsquarters, and wild buckwheat (*Polygonum convolvulus*). Dicamba may also be used at slightly higher rates for control of perennial broadleaf weeds such as Canada thistle (*Cirsium arvense*) and field bindweed (*Convolvulus arvensis*) (Senseman 2007).

Dicamba is absorbed rapidly by most plant tissue including roots, stems, and foliage, but at a slower rate than 2,4-D and other phenoxyacetic acids (Sargent 1976). Dicamba is mobile within plants in both symplastic and apoplastic manners, and utilizes both active transport and passive diffusion to enter cells. Once it reaches the plant's growing points, dicamba accumulates and exhibits prolonged herbicidal activity, though it is degraded by more mechanisms than the phenoxyacetic acids, including hydrolysis, conjugation, and incorporation (Senseman 2007). Dicamba is weakly adsorbed to soil and is rapidly metabolized to CO_2 by soil-dwelling microbes in aerobic soil conditions, and has a soil half-life of under 14 days when soil conditions are moist and warm. However, it may persist longer in soils that do not experience rainfall or moisture, or may leach more readily than 2,4-D if humidity or high-moisture conditions predominate (Senseman 2007). This ability to persist in soils slightly longer than 2,4-D or other auxin herbicides may partially explain dicamba's ability to demonstrate limited residual weed control activity. Lastly, and not surprisingly, the toxicological properties of dicamba are very similar to those of 2,4-D: low acute and chronic toxicity and no reported effects on mutagenicity or reproduction (Senseman 2007).

Herbicide-Resistance to Auxin-Mimic Herbicides

One factor contributing to the longevity of the auxin herbicides is the relatively low incidence of weed species with HR. As of January 2017 there are 33 weed species with reported resistance to synthetic auxins, a relatively low figure compared to other herbicide MOA (Heap 2017). The development of auxinic HR weed species has been slow but steady since its first documentation in wild carrot (*Daucus carota* L.) in Canada in 1957 (Heap 2017; Ryan 1970). Of the 33 species with documented resistance, only 14 have been reported since the new millennium, whereas 15 were reported from 1985 to 1999, despite the steady and heavy use of auxinic herbicides. Mithila et al. (2011) postulate that this low incidence of resistance to the auxinic herbicides can be explained by many factors, including (1) the use of multiple auxinic herbicides tank mixed with herbicides of other MOA, (2) use of cultural practices for weed control in turf such as high seeding rates and frequent mowings and fertilization, (3) the use of high spray volumes and herbicide concentrations to maximize coverage in turf systems, and (4) nonchemical weed control such as hand weeding. Many of these practices, especially mowing and hand weeding, are not feasible in large-scale row crop production, and thus the trend in auxinic HR weed development may be more difficult to minimize when the use of such herbicides increases following the introduction of new crop technology. Additionally, the number of auxinic HR weed species may be underestimated since homeowners and turfgrass system managers do not monitor the occurrence of HR weed species as diligently as row-crop producers (Mithila et al. 2011).

In general, resistance to the majority of herbicides is conferred by a single gene, or in fewer cases two genes (Preston and Mallory-Smith 2001). More specifically, previous work has shown that resistance to dicamba, picloram, and 2,4-D in wild mustard (*Sinapis arvensis* L.) is produced by one dominant gene (Jasieniuk et al. 1995; Jugulam et al. 2005). Additionally,

kochia (*Kochia scoparia* L.) biotypes in Nebraska were shown to have dicamba resistance produced by a single dominant gene (Preston et al. 2009), whereas a single recessive gene controls picloram and clopyralid resistance in yellow starthistle (*Centaurea solstitialis* L.) (Sabba et al. 2003) and quinclorac resistance in false cleavers (*Galium spurium* L.) (Van Eerd et al. 2004). There has been a polygenic source of resistance to MCPA reported in common hempnettle (*Galeopsis tetrahit* L.) by Weinberg et al. (2006), though the majority of reported auxinic herbicide-resistance is conferred by single genes. The monogenic nature of most auxinic herbicide-resistance is cause for concern because single qualitative traits such as those conferring herbicide-resistance spread much more quickly among a population than polygenic traits due to the latter arising from simultaneous manifestations of several mutations, each with low relative probability of occurrence (Jasieniuk et al. 1996; Mithila et al. 2011). Perhaps more unsettling is the fact that the majority of auxinic herbicide-resistance being conferred by single genes is in direct contradiction to previous suggestions by Gressel and Segel (1982) that auxinic herbicide-resistance would require mutations at multiple genetic loci in order to obtain resistance. Mithila et al. (2011) proposes that the low incidence of auxinic HR weed species may be explained by a few factors including (1) the relatively low selection pressure imposed by limited auxinic herbicide use, (2) the transient residual soil activity of auxinic herbicides, (3) the infrequent incidence of resistant alleles in natural populations, and (4) the potential for mutations conferring resistance to be lethal (Jasieniuk et al. 1995). Lastly, many studies have shown that weed biotypes with herbicide-resistance often experience a fitness penalty relative to sensitive biotypes, another potential explanation for the relatively low incidence of resistance to auxinic herbicides (Mithila et al. 2011). Although there is seemingly little incidence of auxin HR weed species, the reality is that the auxin herbicides have not faced the same selective

pressure as other herbicide MOAs, nor has as much attention been paid to the development of auxin-resistant weed species as with other MOAs. Thus, the anticipated widespread adoption of auxin herbicide-based technologies is likely not as secure as it seems.

Effect of 2,4-D and Dicamba on Cotton

In many regions of the country such as in the plains of Texas and Kansas, cotton is often produced near small grains or pasture production systems that include heavy utilization of plant growth regulating herbicides such as the auxin-hormone herbicides 2,4-D and dicamba (Marple et al. 2007). With the rapid development and spread of HR weed species due to the widespread adoption of conservation tillage and subsequent overuse of glyphosate a high adoption of the novel GM row-crop cultivars is expected (Egan et al. 2014; Everitt and Keeling 2007). As such, 2,4-D and dicamba use will increase in many regions, especially those with severe HR weed issues such as Mississippi and the rest of the Mid-South. These herbicides can often unintentionally be misapplied to susceptible crops such as cotton through herbicide drift or tank contamination events, and these misapplication events often correlate with vulnerable early cotton-growth stages (Duncan et al. 1993; Marple et al. 2007 Regehr et al. 2006). While these herbicides are certainly useful in controlling emerged broadleaf weed species, multiple studies show that their potent effects on plant physiology renders them capable of wreaking havoc on susceptible non-target species even when exposure occurs at sub-lethal concentrations (Everitt and Keeling 2007, 2009; Egan et al. 2014; Sciumbato et al. 2004a, 2004b). The off-target movement of herbicides has been a well-documented issue since the inception of modern herbicides (Staten 1946). Previous research has documented the off-target movement of herbicides in many crops ranging from alfalfa (*Medicago sativa* L.) (Al-Khatib et al. 1992) to soybean (Al-Khatib and Peterson 1999) and wheat (Deeds et al. 2006).

The severe impact of 2,4-D on cotton is well-documented, with Staten (1946) reporting cotton injury shortly after 2,4-D's commercialization, and several subsequent studies further characterizing 2,4-D's deleterious effects on cotton crops (Hutchins 1953; Rawson and Schrodter 1981; Regier et al. 1986; Sciumbato et al. 2004a). In general, reports of synthetic auxin herbicide injury on susceptible off target crops are among the most frequently reported complaints in agriculture, largely due to the distinct injury symptoms they cause being easily recognized by producers and land owners (Egan et al. 2014; Marple et al. 2007). The most conspicuous 2,4-D injury symptomology occurs two to five weeks following exposure and includes leaf malformation, epinasty, increased branching, reproductive structure termination, and necrosis (Sciumbato et al. 2004a; Staten 1946; York et al. 2004). However, while auxin herbicide-induced injury is very conspicuous and easy to distinguish, previous work has shown that early-mid season visual injury estimates are a poor indicator of subsequent yield loss as they tend to overestimate the yield loss caused by cotton exposed to dicamba (Byrd et al. 2015; Egan et al. 2014; Marple et al. 2008). Conversely, Egan et al. (2014) showed that cotton visual injury after exposure to 2,4-D can be a relatively reliable indicator of end-of-season yield loss. This disparity is likely due to a differential ability of cotton to metabolize/detoxify dicamba vs. 2,4-D, leading to different rates of recovery from herbicide exposure (Egan et al. 2014; Marple et al. 2008).

Not as much work has been done examining dicamba's effect on cotton growth and yield, ostensibly due to its generally less severe impact relative to that of 2,4-D. Affirming this assumption are results of a 2007 study examining cotton response to various growth regulator herbicides conducted by Marple et al., which found that fiber yield was reduced most severely by all formulations of 2,4-D followed by picloram and fluroxypyr, and to a lesser extent,

dicamba. Egan et al. (2014) performed an extensive analysis of existing research on cotton's response to 2,4-D and dicamba, and reconfirmed that cotton is much more susceptible to 2,4-D than dicamba in general, and is in fact more sensitive to 2,4-D than soybeans are to dicamba. Although it is clear that susceptible cotton is severely affected by exposure to 2,4-D and to a lesser extent dicamba, it is important to note that its response to these herbicides is dependent on a plethora of factors including moisture and climate, stage at the time of exposure, length of the growing season (impacts the amount of time for the crop to recover), specific cultivar genetics, herbicide carrier volume, herbicide formulation, and any combination of these and other unlisted factors (Byrd et al. 2015; Egan et al. 2014; Marple et a. 2007).

Effect of Cotton Growth Stage on Susceptibility to 2,4-D and Dicamba Exposure

In general, young cotton is most susceptible to herbicide injury (Duncan et al. 1993; Regehr et al. 2006). Everitt et al. (2005) found that the greatest cotton yield loss occurs from exposure at the pinhead square growth stage. Similarly, Hamilton and Arle (1979) showed that low rates of dicamba reduced cotton yields more severely when exposure occurred prebloom versus at or after bloom. In similar studies conducted with sub-lethal rates of picloram, triclopyr, and clopyralid, Jacoby et al. (1990) showed that prebloom exposure had more severe effects on cotton yield loss than postbloom exposure. The importance of exposure timing on determining cotton response to PGR herbicides was further documented by a 1991 study by Snipes et al., which showed that cotton response to triclopyr was determined more by exposure timing than triclopyr rate.

As the risk of cotton exposure to 2,4-D and dicamba has increased with the pending registration of auxin-tolerant row crop cultivars, more studies have further characterized cotton's varying response to sub-lethal rates of the auxinic herbicides as a function of growth stage at the time of

exposure. Using rates of 1/2, 1/20, 1/200, and 1/2000 of the recommended use rates of 0.56 kg ai ha^{-1} for 2,4-D and 0.28 kg ai ha^{-1} for dicamba, Everitt and Keeling (2009) showed that dicamba reduces cotton yield more severely when exposure occurs at the early blooming growth stage, whereas 2,4-D has severe effects on yield when exposure events occur either at PHSQ or the cotyledon-two leaf stage. Further confirming these implications are a robust 2014 study by Egan et al. which analyzed the results of 15 experiments studying dicamba's effect on cotton at various growth stages and 48 such studies with 2,4-D. This study found that young flowering cotton is most susceptible to low concentrations of dicamba whereas vegetative or preflower squaring cotton was most susceptible to sub-lethal rates of 2,4-D (Egan et al. 2014). In 2015, Byrd et al. showed somewhat contrasting results in a study that tested cotton's response to two sub-lethal rates (a drift rate of 2 g ae ha^{-1} and a contamination rate of 40 g ae ha^{-1}) at the four-leaf, nine-leaf, full-bloom (FB), FB+2 week (wk), FB+4 wk, and FB+6 wk growth stages. The results of this study indicated that the most susceptible cotton growth stage varied based on whether exposure was to a drift or contamination rate and on environmental conditions, with the author citing temporal differences in the shift from vegetative to reproductive growth as causing variability in cotton response (Byrd et al. 2015). Additionally, previous work by Marple et al. (2007) concluded that the severity of visual injury caused by early-season exposure to 2,4-D or dicamba is not necessarily indicative of corresponding yield loss severity, and that late-season injury is a better indicator of yield loss. This variability in the relationship between visual injury and subsequent yield loss is likely explained by cotton's differential ability to recover from herbicide injury and manifest compensatory yield, as Sawchuk et al. noted with canola (*Brassica napus*) and white bean (*Phaseolus vulgaris*) in 2006.

Future Outlook on 2,4-D and Dicamba Use

The commercialization and implementation of crop cultivars with engineered tolerance to POST applications of 2,4-D or dicamba will add a much-needed tool to the weed control arsenal as producers are faced with increasingly difficult-to-control weed species. Upon release and full registration of all components of these weed control technologies, it is anticipated that the rate of their adoption will be quite high. While this may be intuitive given the advantage they will provide to controlling HR weed species, there will be several additional pressures to adopt these new technologies, including: (1) most broadleaf crops are highly sensitive to auxinic herbicides even when exposure is to a diluted amount, (2) as most producers depend on commercial applicators to apply herbicides the chances of misapplication events is high given that the same applicator may service both susceptible crops and those with GM auxinic HR traits, (3) it has been shown that auxin herbicides can be exceedingly difficult to remove from spray equipment, (4) various formulations of auxin herbicide products have been shown to have high volatility and thus a propensity for herbicide drift, and (5) the consolidation of the seed and agrichemical industries leaves producers with fewer options for high-yielding cultivars with conventional genetics (Behrens and Lueschen 1979; Boerboom 2004; Cundiff et al. 2017; Everitt and Keeling 2009; Mortensen et al. 2012). As such, a high rate of adoption of the new GM technologies is anticipated.

However, the concerns associated with the use of such auxin-mimic herbicides will become more prevalent with their increased use. Auxin herbicide drift has been well documented and substantiated over the long use history of 2,4-D and dicamba (Behrens and Lueschen 1979; Bovey and Meyer 1981). Adding to drift concerns is the penchant for some auxin-herbicide formulations to be prone to volatilization, which produces driftable herbicide vapor droplets (Behrens and Lueschen 1979). In fact, a 1990 study by Taylor and Spencer demonstrated that

volatilization can be responsible for the removal of up to 90% of an applied herbicide, depending on environmental conditions. Concerns about herbicide volatility may be somewhat mitigated by the development of new 2,4-D and dicamba formulations that contain volatility and drift-retardant technology. However, the possibility for herbicide drift remains high, as labels for the new product formulations will allow application at up to 24 kilometers per hour (KPH), and history dictates that producers are not always judicious in their use of herbicides and may choose to illegally use older, cheaper auxin herbicide formulations with greater rates of volatilization (Egan et a. 2014). Additionally, two studies have shown that in geographic regions with heavy 2,4-D and dicamba use, herbicide residues may actually accumulate in the atmosphere and subsequently precipitate onto non-target fields at rates high enough to induce crop injury (Hill et al. 2002; Tuduri et al. 2006). Further complicating the issue is the tendency for synthetic auxin herbicides to persist in herbicide spray tanks and other equipment such as hoses and spray nozzles (Boerboom 2004; Cundiff et al. 2017). Johnson et al. (2012) tested cotton's response to low concentrations (such as would arise in a tank contamination event) of 1/2, 1/8, 1/32, and 1/512 the manufacturer's suggested use rates of 280 g ai ha^{-1} dicamba and 540 g ai ha^{-1} 2,4-D amine, and noted a yield reduction in three of four site-years from all rates of 2,4-D, and in only one site-year from each of the two highest rates of dicamba. Marple et al. (2008) also found that sub-lethal rates of both 2,4-D and, to a lesser extent, dicamba reduced yield. Thus, the many possible vectors of off-target movement of the auxinic herbicides poses a potentially serious threat to susceptible crops. This threat is somewhat magnified by the fact that in most of its region of production in the US, cotton is planted before soybeans and other row crops that will utilize these novel auxin herbicide technologies, resulting in a higher chance of cotton exposure to misapplication events arising during herbicide burndown applications in

later-planted crops (Egan et al. 2014). Additionally, current and anticipated herbicide management programs involve the use of multiple applications that can occur throughout the growing season. Thus, the possibility of misapplication events occurring remains throughout the growing season.

Risk of Development of Resistance to Auxin Herbicides

The new weed control technologies will face the same possibility of the development of HR weed species that befell other chemistries such as the triazines, ALS-inhibitors, and glyphosate. As such, it will be important to properly steward these new technologies to protect their longevity and efficacy. To that end, many studies have been conducted to determine best management principles for using herbicides to control weeds. Previous research has shown that the use of herbicides with multiple MOA in tandem and in rotation helps stave off the development of herbicide resistance (Beckie and Reboud 2009; Gressel and Segel 1990; Powles et al. 1997; Wrubel and Gressel 1994). Tank-mixing two herbicides can add up to four years before the onset of herbicide resistance development, as compared to rotating herbicides each year (Powles et al. 1997). Neve et al. (2003) simulated the development of HR weed species in a 100 ha^2 area with a beginning seed bank of 100 seeds m^2 and resistant gene frequency of 10^6 and found that resistance developing to each of two herbicides used as a tank-mix was unlikely within 50 years for all weeds, especially if measures were taken to minimize the spread of weed tissue. Furthermore, the study found that when the herbicides were used in alternate years (rotating) multiple resistance arose in nearly all 100 ha^2 areas (Neve et al. 2003).

HR weeds pose a significant challenge to crop producers, one that the new auxinic herbicide weed control systems hope to address. However, the auxin herbicides 2,4-D and dicamba are at just as much risk for an increase in HR development as any other chemistries, as resistance to

each has already been confirmed in some species (Heap 2017). While 2,4-D and dicamba have great promise in dealing with difficult weed species, they are not the silver bullet in and of themselves, as each struggles to control certain broadleaf weed species and have little control on grass weed species. Additionally, the auxinic herbicides can sometimes reduce the efficacy of POST grass herbicides, further confounding their role in weed control (Mithila et al. 2011). Fortunately, however, Johnson et al. (2010) found that tank mixing dicamba with glyphosate provided high levels of control of GR weed species.

Ultimately, the new weed control technology will provide another tool for controlling HR weed species and by learning from previous lessons and using the approaches previously discussed including taking an integrated approach to weed management that includes biological, cultural, physical, and chemical inputs, the longevity and efficacy of these new technologies can be maximized. As Mortensen et al. (2012) posit, contrary to prevailing industry opinion, the potential for auxinic HR weed species developing following the predicted future adoption of auxin-based technology could actually be quite high and thus a strategic and conservative approach to utilizing these new herbicide technologies will be necessary in order to avoid the risk of exacerbating the HR weed problem in the long run.

Tables

Table A.1 Recent 2,4-D and dicamba use trends in major row crops.[a]

Year	Crop	Total domestic 2,4-D used	Total domestic dicamba used
		kg 2,4-D ae	kg dicamba ae
2015	Winter Wheat	2,108,749	438,170
2015	Spring Wheat	397,347	51,709
2015	Cotton	309,803	58,967
2015	Oats	117,934	4,536
2015	Soybeans	3,499,462	78,018
2014	Corn	1,919,148	591,484
2013	Peanuts	94,347	--
2013	Rice	60,781	--
	Total	8,507,572	1,222,884

[a]Source: USDA-NASS 2016

Figures

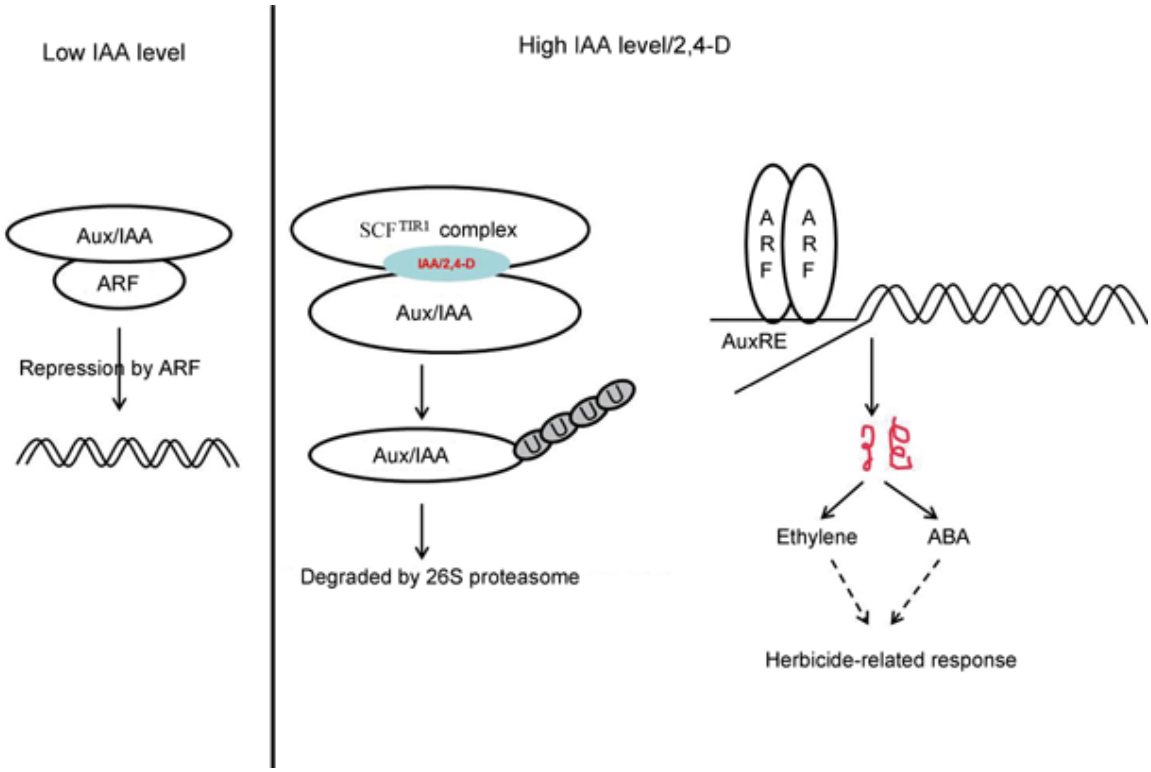

Figure A.1 Suspected physiological effects of low (left) and high (right) levels of IAA or IAA analogs[a*].

[a] Source: Song 2014
[*] IAA: Indole-3-acetic acid; Aux/IAA: Auxin/IAA active repressor protein complex; ARF: Auxin Response Factor; SCF^{TIR1}: Skp, Cullin, F-box containing complex containing the TIR1 F-box protein

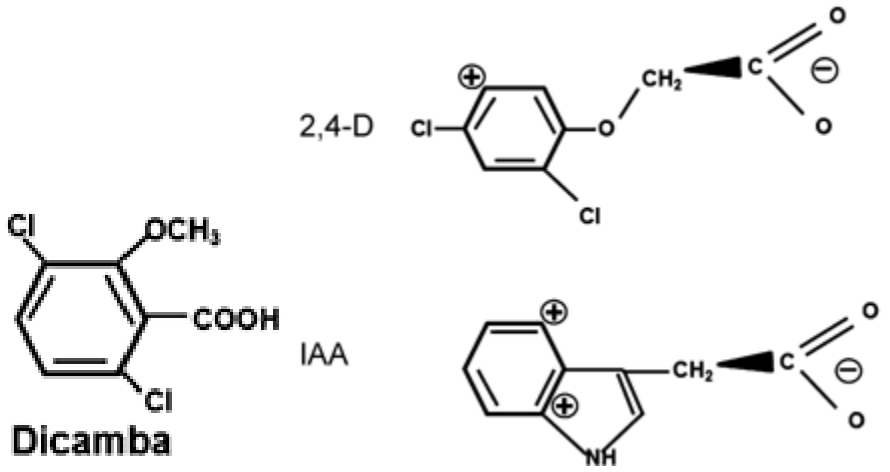

Figure A.2 2,4-D (top) and dicamba (bottom left) are structural analogs of the native plant hormone indole-3-acetic acid (bottom center) which allows herbicidal activitya

[a]Source: Song 2014

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