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Investigating Dicamba Tolerance in Grapevine Cultivars through Drift Simulation Assays

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**INVESTIGATING DICAMBA TOLERANCE IN GRAPEVINE CULTIVARS
THROUGH DRIFT SIMULATION ASSAYS**

A Master's Thesis

Presented to

The Graduate College of
Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree
Master of Science, Plant Science

By

Bryce D. Bentley

December 2019

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Plant Science

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Master of Science

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ABSTRACT

Dicamba is a synthetic auxin herbicide which acts systematically and selectively controls broadleaf plants and has become increasingly popular in the past several years for weed control in commercial agriculture following widespread adoption of dicamba-resistant, transgenic soybean and cotton. Dicamba may be better acknowledged by growers of specialty crops, like grapes, for its capacity to drift miles away from the site of application and deposit on fields, remaining potent enough to cause crop damage and yield loss to vulnerable plants. Grapes are among the most susceptible agronomic crops to dicamba drift, showing visible injury at rates less than 1% of standard commercial applications. To counter the threat dicamba poses to the grape and wine industry, this study investigates the potential for the *Vitis* interspecific hybrid chambourcin to be utilized as a source of resistance to dicamba in future grape breeding efforts. Few reports on dicamba tolerance in grapes exist; however, previous field studies have implicated chambourcin as resistant. chambourcin and proposed susceptible cultivar *Vitis vinifera* cabernet sauvignon will be subject to sub-lethal, spray drift simulating treatments of dicamba as well as lab-based detached leaf assays that mimic the vapor drift of dicamba while simultaneously mitigating the effect of confounding environmental factors. Injury status will be determined *in vivo* by tracking shoot length to several weeks after treatment and by visual assessment of dicamba's distinctive leaf cupping damage and stem and petiole distortion. *In vitro*, injury will be observed through leaf cupping and necrosis. The goal of these experiments is not only to determine the differential effects of dicamba drift on these two grapevine cultivars, but to produce valid and replicable experimental protocols by which to assess the severity of dicamba drift damage in any grapevine cultivar.

KEYWORDS: dicamba, synthetic auxin, grapevine, herbicide drift, simulated drift, vapor drift, chambourcin, cabernet sauvignon, vitis vinifera, herbicide resistance

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

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INTRODUCTION

US agriculture has come to rely on the use of chemical pesticides to suppress weed proliferation in the production of row crops. In recent years, the popularity of genetically engineered (GE) crops modified with herbicide resistance traits has further established this trend of herbicide use in US cropping systems. This system has shown success in controlling unwanted plants in the field, however after many seasons of use, large populations of weed species are showing resistance to these ubiquitously utilized herbicides. In the newest solution to this crisis, resistant weeds are now combated with transgenic crops engineered with two or more herbicide resistance genes combined, or “stacked”, together in the genome of one single plant. These new genetically engineered crops make use of old herbicides, such as dicamba, for which relatively few plants currently demonstrate resistance, to better control the adapted weed populations. Dicamba has previously been used as a pre-plant and pre-emergent herbicide with limited application across the country. Annual rates of applied dicamba in the US have increased from five million pounds a year to no less than twenty million, and is likely to be substantially higher in years to come (Cox, 1994; USDA, 2014). Large volumes of dicamba are applied later in the growing season as a post-emergent herbicide under widespread use across the nation. This idea is controversial as later in the season when temperatures rise, dicamba’s volatile nature allows it to more readily vaporize and move offsite where it may then damage susceptible crops in its wake (Behrens and Lueschen, 1979). It doesn’t help that this herbicide is highly toxic to many different agronomic crops including tobacco, tomatoes, legumes, and grapes. It can be argued that vineyards are most at economic risk from the mass implementation of dicamba use in row crops due to the perennial nature and long production cycles of the grapevine. Currently, no

grapevine variety has been commercially marketed for its ability to tolerate dicamba injury, and no known genes or DNA markers related to this characteristic have been identified in grapes, yet such a trait could still exist. The goal of this project is to investigate claims that French-American hybrid grapevine cultivar chambourcin has an elevated tolerance to dicamba drift and its subsequent injury by testing it against the proposed dicamba-sensitive cultivar cabernet sauvignon. Two separate dicamba assays are performed repeatedly between Fall 2018 and Summer 2019 to refine protocols used to detect phenotypic responses from either cultivar. Spray drift simulating foliar applications of dicamba target young vines to produce sub-lethal dicamba injury conditions *in vivo* in a greenhouse study, while an *in vitro* detached leaf assay attempts to elicit dicamba injury in detached grape leaves through subjection to vapor drift. These tests investigate at what dicamba concentrations and timepoints after dicamba exposure that injury begins to occur, and how it develops in severity over time in different grapevine varieties. Concurrent on finding a dicamba tolerance trait between chambourcin and cabernet sauvignon, future research will investigate a hybrid population between chambourcin and cabernet sauvignon to determine the heritability of dicamba tolerance, and find DNA markers associated with this trait. These DNA markers could be key to breeding future novel varieties of dicamba tolerant grapevine through the use of marker assisted selection technologies.

Background and Literature Review

Importance of Pesticide Use in Agriculture. Modern agriculture relies heavily on the use of chemical herbicides to lessen the economic impact of weed species in the row crops. Following World War II and the commercial release of several different pesticides, commercial agriculture and herbicide use have maintained a strong relationship (Gianessi and Reigner,

2007). Today, with decades of research in integrated pest management, weed control systems can be designed to tackle the problem of unwanted plant species using an economical blend of preventative measures, cultural practices, mechanical control, biological control, and chemical control (Oregon State University Forage Information System, a). Still, chemical pesticide use cannot seem to be replaced in agriculture; without herbicide use, crop yields would decline 20%; to fully substitute the effectiveness of herbicides through tillage and hand-weeding, an estimated 70,000,000 additional workers would be required in the US (Gianessi and Rieger, 2007). Chemical pesticides are relatively cheap and easy to implement in cropping arrangements compared to more labor-intensive weed eradication programs (Panozzo et al, 2015). In 2017, US farmers produced just shy of 200 billion dollars' worth of crops after investing less than sixteen billion dollars on pesticide applications (USDA ERS, 2019a).

Herbicide Tolerance Traits in Genetically Engineered Crops. The most alluring pesticides to use in production agriculture are ones that have selective modes of action. Pesticides that act in a non-selective herbicidal capacity will indiscriminately injure all plant types, whereas selective herbicides affect specific processes that only some plants possess (Oregon State University Forage Information System, b). As such, producers can apply specific herbicides directly over crops during the growing season to eliminate weeds without damaging crops. The introduction and widespread adoption of herbicide resistant, or herbicide tolerant (HT), GE crops in agriculture in recent decades similarly utilizes this principle. Crops are modified at the molecular level to no longer be selected for by specific herbicides. These plants have been bioengineered to include transgenes which can detoxify or otherwise render herbicides harmless within plant tissues. The allure of these GE crops that they effectively prevent herbicides from affecting, or selecting for them, making it possible to kill weeds with herbicides

which typically harm the crop. In HT GE cropping systems, crops which are typically susceptible to specific herbicides are transformed to resist this damage with genetic modification. Thus, sprays of herbicides on HT crops, whether selective or non-selective in nature, are made possible without catastrophic crop injury or loss. The idea behind these genetic modifications is to provide simple, reliable, and cost-efficient weed control in the transformed crops, as well as providing a wider array of herbicidal options for producers whom were limited in the past.

Examples of this HT GE technology include glyphosate-resistance conferred to soybeans, first introduced to the market by Monsanto (St. Louis, MO.) in 1996, and later corn in 1998 through genetic modification. Glyphosate [N-(phosphonomethyl)glycine] is a non-selective herbicide, discovered in 1970 by Monsanto, which disrupts a key enzyme in the shikimate biosynthetic pathway responsible for production of the amino acids tryptophan, tyrosine, and phenylalanine. According to the Weed Science Society of America (WSSA) handbook, 9th ed., this disruption prevents the formation of the essential plant cell component lignin, and several other substantive plant elements (Green, 2009). Glyphosate can control virtually all plant types and has been the most widely used herbicide in the US since 2001 (Livingston et al, 2015; Henderson et al, 2010). At least nine glyphosate resistant GE crops have been created worldwide, including soybean, corn, cotton, two varieties of canola, alfalfa, sugarbeet, creeping bentgrass, and wheat (ISAAA, 2019). These crops are all designed to express a gene which breaks down the glyphosate into less phytotoxic metabolites (Shaner, 2014). In 2008 alone, growing acres spread across more than 114 million growing hectares (282 million acres) were sprayed with glyphosate in 23 different countries. While glyphosate resistance is the most recognizable GE trait implemented thus far, other herbicides, such as glufosinate, have been used in HT GE technologies to meet a similar fate. The swift, widespread acceptance of this

technology among farmers indicates that future GE crop technologies are adopted quickly by US farmers (Green, 2009). The use of such herbicide-tolerant GE crops is nearly ubiquitous in US agriculture; plateauing at 94% of planted acres of corn and soybean in 2014 (USDA ERS, 2019b).

Emergence and Effect of Herbicide Resistant Weed Populations. Unfortunately, the benefits of these herbicide-resistance GE technologies do not persist indefinitely at their initial effectiveness. The continuous use and reliance upon specific herbicides or specific modes of action by growers to combat plant pests has decreased the efficacy of many of these chemicals by promoting the introgression of herbicide resistance into weed populations. (Shaner, 2014). As of 2015, at least 236 weed species worldwide have been identified to have developed resistance to at least one herbicidal mode of action, restricting the options for producers to combat problem weeds. Herbicides revolutionized agriculture, but eventually selected for the growth of herbicide resistant weed populations (Panozzo et al, 2015; Shaner, 2014). Detailed by Cerdeira and Duke (2006, cited by Behrens et al, 2007), the rising populations of herbicide resistance weeds will pose a threat to the viability of no- and reduced-till operations designed to protect soil health and erosion, as the currently rely heavily on pre-plant herbicide applications for weed control.

In air of the ubiquity of glyphosate resistant GE cropping systems, farmers began noticing increasing populations of glyphosate resistant weed species. 2012 USDA figures revealed that greater than 5% of corn acres were infested with glyphosate resistant crops, and 40% of soybean acres showed a reduced effectiveness of glyphosate application in weed control during the 2012 growing season (Livingston et al, 2015). In less than 20 years after their commercial debut, these GE cropping systems have become the victim of their own demise. In the nations that quickly converted their agriculture systems to rely heavily on the use of a select

few herbicides, HT weed species pose a substantial threat to the agrarian economy as well as food security.

Implementation of Stacked Herbicide Resistance Traits in Agronomic Crops. The reality brought on by the enduring emergence of HT weed populations continues to push biotech firms towards further innovation, creating newer generations of HT GE crops. After the commercial success of early GE crops, biotechnology firms did not cease research on genes of interest which could be implemented in future renditions of GE crops. As a result, many genetic traits, such as new herbicide tolerance, have been located across genomes and could easily be implemented within new GE crops (Green, 2009). Instead of basing new GE cropping systems solely on these freshly uncovered traits, there is an industry trend referred to as gene pyramiding, or “stacking”, where several transgenic traits which combine one or more different advantageous traits within one plant. This trend is not likely to die down; in 2012, 26% of GE crops included 2 or more transgenic modifications, while in 2018, greater than 80% of total corn and soybean plants were grown with stacked transgenes (James, 2012; USDA ERS, 2019b). Even when one herbicide begins to lose effectiveness in single HT gene GE systems, such as occurred with glyphosate, other HT genes can be used as a supplemental protection from multiple herbicides. Several biotech firms have gone this route, combining one or more HT genes within on specific crop, allowing them to be sprayed with a combination of herbicides which previously would have been lethal. Examples of such crop varieties include Bayer’s Roundup Ready 2 Xtend™ soybeans resistant to glyphosate and dicamba (3,6-Dichloro-2-methoxybenzoic acid), their XtendFlex™ cotton series which boasts resistance to glyphosate, dicamba, and glufosinate (2-amino-4-methylphosphinobutyric acid); DuPont’s Enlist™ soybean, corn, and cotton varieties which all resist combination 2,4-D (2,4-Dichlorophenoxyacetic acid) choline and glyphosate

applications (Enlist Weed Control System, 2019; roundupreadyxtend.com, 2019). These new GE crops hold promise for their ability to eradicate glyphosate-resistant weeds that remain susceptible to 2,4-D and dicamba, but since these herbicides have never been implemented in such a widespread manner as these new systems call for, the unintended consequences are largely unknown.

Dicamba

Herbicidal activity produced by dicamba is potent. While its use in GE crops is relatively new, the chemical has been commercially available for over 50 years (Gianessi and Reigner, 2007). Dicamba is a selective herbicide in the chlorophenoxy family (Bunch et. al, 2012). These herbicides are generally known for their low mammalian toxicity; pasture-fed livestock cannot achieve intake of fatal doses after grazing a sprayed field (Gupta and Crissman, 2013). Dicamba is a class 4 pesticide, falling with the other plant growth regulators. Specifically, similar to 2, 4-D, dicamba is a synthetic auxin herbicide which target plants by mimicking the phytohormone auxin within plant growth signaling pathways. Auxin is naturally occurring within plant tissue and typically exists as indole-3-acetic acid (IAA). IAA is responsible for many of the factors relating to plant growth and development. Within the plant cell, IAA breaks down by undergoing cellular processes where it quickly becomes inert. However, synthetic auxin herbicides cannot be broken down readily in most plants, persist for longer durations, and lead to a more potent effect on plant signaling than natural IAA (Grossmann, 2007). The half-life of IAA proteins in plants is short, typically six to eight minutes (Eckardt, 2001). In contrast, the half-life of dicamba is between four and twenty days on average between 153 plant species sampled in 2013 (Fantke et al, 2014; WSSA, 2018). The half-life in susceptible plants is likely higher than in more resistant

ones. Dicamba tends to accumulate in meristematic tissue of the young leaves and shoot tips (Chang and Vanden Born, 1971; Magalhaes et al, 1968). The complete mechanism of action of this herbicide is largely unknown, although it is thought to “result from uncontrolled cell and tissue proliferation” (Cranston et al, 2001), which leads to plant death.

Effect of Dicamba Formulation, Environment, and Other Factors on Drift Injury.

Dicamba is infamous for how readily it seems to cause susceptible crops unintended damage. Many high value crops are extremely sensitive to dicamba. The University of Georgia at Tifton compiled data from field trials and literature and found at least seven crop varieties which show extreme sensitivity to dicamba, with visual injury occurring at less than 1/800 of the typical application rate. These crops include grapevine, lima bean, southern pea, snap bean, soybean, sweet potato, and tobacco (Culpepper et al, 2017). University of Arkansas weed scientist Jason Norsworthy reported “seeing more than 10% [dicamba] injury to soybeans” with atmospheric concentrations as little as one nanogram per cubic meter, or one part per quadrillion (Unglesbee, 2019a). With such miniscule rates creating visible crop damage on a variety of agronomic crops, producers of sensitive crops are weary of how widespread adoption of dicamba will affect their livelihood. Most damage to susceptible crops results from the phenomenon known as herbicide drift, which is characterized by the unintended, off-target movement of an herbicide away from the site of application or any other source of the chemical. Damage from drift typically occurs either as spray drift where particles remain airborne after application and overshoot their target, or as vapor drift where an herbicide evaporates and is then carried away from treated plant and soil surfaces. Dicamba is well known to form vapor post-application due to its high vapor pressure (Gullickson, 2019), and has the potential to travel up to 10 miles under specific conditions (Cox, 1994). There are more than a dozen considerations to take when spraying

dicamba to avoid drift. These factors include the formulation of dicamba, wind direction, wind speed, buffer distance between crops, time of day, type of nozzles, spray tank additives, boom height, travel speed, and presence of a temperature inversion in the environment. For newer dicamba formulations, which are the only herbicides registered for use with new GE crops, applicators are legally required to adhere to strict guidelines relating to these factors. Even under idyllic conditions where all label recommendations are followed, offsite movement of dicamba is possible (Gullickson, 2019).

Another means by which accidental dicamba damage can occur is through insufficient cleaning of the spray tank and path on application equipment (Boerboom, 2004). It seems that only 80% of residual dicamba is removed during each successful spray tank rinse. Even after the recommended triple rinse of tanks with ammonia-water solutions, it is still possible for dicamba to remain potent enough to injure and affect the yield of tobacco plants (Inman et al, 2014). Of course, nobody is trying to cause damage to their crops, or in neighboring fields' crops, but damage still occurs.

Reports of dicamba drift damage have existed for as long as the chemical has been available commercially, and may worsen in the future. Many producers of susceptible crops in the Eastern US are able to recognize the distinctive signs of dicamba drift damage on their plants; in the past, the Association of American Pesticide Control Officers (AAPCO) has ranked dicamba consistently among the top chemicals responsible for crop-injury complaints dating back to the 1960s (Egan et al, 2014). Of course, this tracks data from before the advent of dicamba-tolerant GE crops. Historically, dicamba has acted primarily as a pre-plant and pre-emergent herbicide, used to control broadleaf weeds in the corn industry, in non-leguminous forage and pasture crops, as well as in the turf and lawn industry. Dicamba use was relatively

low volume, with limited sprays occurring post-emergence. Not only will new dicamba resistant GE crops call for greater volumes of dicamba to be used, but also new labels call for use post-emergence. This means dicamba can be sprayed over the tops of actively growing soybean, cotton, and corn fields (Egan et al, 2014). Spraying dicamba in this fashion will likely lead to more off-target drift complaints, as plants are generally more sensitive to synthetic auxins during periods of intense growth. Later in the season also sees greater chances for high temperatures and temperature inversions, and the associated increased risk of vapor drift injury. This situation is characterized when elevated temperatures during the day turn into evenings with inverted temperatures, where layers of hot air smother stagnant cool air layers. Under these conditions, vapor from recently applied dicamba can be held close to the earth for a long duration where it may cause injury to susceptible plants. While applicators are obligated to restrict sprays to cooler days, this can prove to be difficult. It also turns out that temperature inversions are more common than once believed: a multi-year study by the University of Missouri showed that temperature inversion occurred on more than 60% of evenings in June and July 2018 (Bish and Bradley, 2019). While producers do their best to comply with stringent label requirements, too often spray conditions are less than ideal. Despite the clear downsides of increasing dicamba spray volume later in the season, biotech firms are still making a push towards adoption of these technologies.

Timeline of Dicamba Resistant GE Crops. According to Reuter’s “The Seeds of Monsanto’s weed-killer crisis”, Monsanto first licensed their genetic trait responsible for dicamba resistance and began breeding these traits into soybean and cotton. Concerns about dicamba volatilization and drift injury of sensitive crops were voiced as early as 2011 by researchers of Ohio State University, yet Monsanto would go on in 2012 to state their plans to

make all future varieties of soybean and cotton resistant to dicamba. To limit the effect of dicamba volatility, Monsanto concurrently designed a low volatility formula of dicamba for use alongside future crop lines. Monsanto sought EPA approval of this herbicide in 2013, but this would not be granted until November 2016. Prior to this EPA approval, Monsanto made the decision to have limited releases of their new lines in 2015 and 2016. Despite instructions to not perform any illegal sprays of older dicamba formulas on these new varieties, many producers chose to ignore these directions. Increased dicamba injury complaints on soybeans were first reported during 2015, then saw a five-fold increase in the 2016 season. In the 2017 season, with Monsanto's XtendiMax® herbicide with VaporGrip® Technology, and several other brand's low volatility dicamba formulas finally approved for use, dicamba drift complaints were expected to be lower than the previous season. Unfortunately, dicamba drift complaints continued to ring in across the country. In 2017, dicamba drift injury was severe enough for at least two states, Missouri and Arkansas, to temporarily ban all dicamba products in July of that year, imposing fines up to \$25,000 per incidence of misuse (Davies, 2017). Whether the drift was due to lawful applications of new dicamba formulas, or more illegal uses of older formulas is up in the air, however the EPA still decided to place these new formulations under review. This led to new regulations around spraying dicamba that would be implemented for the 2018 and 2019 growing seasons (Flitter, 2018). New rules impose smaller time windows to spray each day and tighter recordkeeping deadlines, limit sprays to 45 or fewer days after planting soybean (60 for cotton), make applicator certification a must for sprays, require more strenuous cleanout procedures, specify nozzles for application, and delegate the use of adjuvants or tank mixers to reduce vapor formation. New requirements also mandate applicators receive proper training on the use of auxinic herbicides (Holder, 2019). Despite these efforts, dicamba drift complaints have persisted.

In Illinois, the US's largest soybean producer, total dicamba injury complaints increased from 2017 and 2019. 2017 saw 246 complaints statewide; 330 in 2018; then 590 in 2019 (Nosowitz, 2019).

Concerns about Low Volatility Dicamba Formulations. The greatest fears of implementing dicamba resistant GE cropping systems come from the potential for off-target movement of dicamba vapor. These fears were initially quenched when Monsanto said only low-volatility formulas of dicamba would be used with their Roundup Ready 2 Xtend® soybeans and Bollgard II® XtendFlex® cotton. Volatility figures for Monsanto's XtendiMax® herbicide with VaporGrip® Technology claim vapor drift is reduced 90% from the diglycolamine (DGA) salt of dicamba and is about 400 times lower than dimethylamine (DMA) salt of dicamba (Roundupreadyxtend.com, 2019). Other low volatilization dicamba products from other firms, such as Engenia® by BASF, FeXapan® herbicide Plus VaporGrip® Technology by DuPont, and most recently Tavium® Plus VaporGrip® Technology by Syngenta, boast similar results. One major concern which has come up in recent years is how effective these formulas remain under real-world conditions, such as when mixed with other herbicides, including glyphosate. In previous spray-drift experiments performed at Ohio State University, dicamba has been shown to elicit more severe herbicidal damage in specialty crops when combined with glyphosate versus when either herbicide is used alone (Wolfe, 2013). Other studies have similarly implicated dicamba to volatilize to a greater extent when mixed at low pH, such as with glyphosate (Holder, 2019). One study investigating an older formula of dicamba (DGA) alongside the newest low volatility formula (Vaporgrip®), performed at the University of Tennessee, demonstrated that dicamba has greater levels of volatility when mixed with glyphosate. The study suggests that glyphosate additions can increase volatility of Vaporgrip® formulas by an average of 3.3 times

compared to Vaporgrip® alone. More interesting, however, are the findings that volatility between DGA plus glyphosate and Vaporgrip® plus glyphosate differ by just 40% (Mueller and Steckel, 2019). It seems that not only does the addition of glyphosate to dicamba increase its volatility, but also negates most of the benefit of said low volatility formulations.

Combating Dicamba Drift. As dicamba vapor has the capacity to travel for miles (Cox, 1994), and can take between 10 and 21 days for exposure symptoms to appear on soybeans (Loux and Johnson, 2017) and potentially longer in other crops, finding the source-point for any given drift incidence may be difficult, if not impossible. After the herbicide injury has appeared, the damage has already been done, and producers of vulnerable crops are left with few options. Producers may choose to file a liability crop insurance claim and risk entering polarizing lawsuits with neighbors, or to simply eat the damage and start fresh next season. It is important to note that dicamba drift damage is not federally insured; thus, producers require additional liability coverage from separate entities which may be able to provide yield loss coverage, given the source of dicamba can be pinpointed (Grinnell Mutual, 2019). Even in cases where these chemical sources can be pinpointed, insurance assessors may conclude that the dicamba applicator lawfully applied the product to their crops and are therefore not responsible for any drift damage, meaning that specialty crop producers may face “financial losses with no compensation” (Unglesbee, 2018). Due to the difficulties involved in dealing with dicamba damage retroactively, producers have more success when opting to take preventative measures. For soy and cotton growers, the best preventative measure to avoid future dicamba drift injury in their fields would be to adopt dicamba-resistant cropping systems. themselves Unfortunately, this adoption would lead to increased dicamba drift for specialty crop producers who do not have the ability to grow dicamba resistant varieties. These producers’ preventative measures are much

more limited. The best method for these producers to avoid dicamba drift would be to select a site at least a dozen miles away from sites where dicamba may be utilized, however this may be impossible for the thousands of specialty crop growers in the Midwest and eastern US. The next best, and more realistic, option for these producers is to bring awareness to the vulnerability and location of their specialty crops to neighbors. In some cases, simply talking to one's neighbors and discussing plans of action to introduce vegetative buffers, or to reduce sprays in certain areas during sensitive growth stages may greatly reduce the impact of dicamba drift. Different programs, such as DriftWatch by FieldWatch, have been created for such purposes. With DriftWatch, producers can voluntarily register the location of sensitive crops for any pesticide applicators to see and aims to heighten the cooperation and collaboration between dicamba applicators and those most in danger of its drift to prevent and manage off-target injury (Driftwatch.org, 2019). While these voluntary programs may help to mitigate dicamba drift damage, the ability of producers to reduce the impact of dicamba drift is often out of their hands. The uncontrollability and unpredictability of dicamba drift may simply lead to it being another issue with which producers and consumers alike to account for perennially. The opportunity cost of widespread dicamba usage could possibly be the relative abundance and quality of the many specialty goods affected by dicamba. While supply chains for certain specialty crops may be impacted, this new paradigm could also open up avenues for new regions, less affected by dicamba, to become key production regions for these marginalized specialty crops if dicamba drift persists.

Dicamba and Grapevines

Unlike many of the other specialty crops highly sensitive to dicamba, grapevines are perennially maintained, and have substantial economic impacts across the regions afflicted with dicamba. Combining the long-lived nature of grapevines with their high cash value per acre and their extreme sensitivity to dicamba, it can be argued that vineyard owners are most at risk from the widespread use of dicamba. Growing grapes is a long-term investment. Typically, grapevines are not harvested until their fourth or fifth season and reach maximum productivity around 25 to 30 years of age (Considine, 2008), although can remain productive for much longer. While young vines are more sensitive than established ones, dicamba drift can affect any vine. Even single exposure events to low rates of dicamba have the potential to affect the berry set of adult vines in subsequent years (Nebraska Winery & Grape Growers Association, 2012). In cases where vines are not killed or severely stunted by dicamba drift, the cumulative effect of multiple drift events within and across seasons may lead producers to prematurely remove vines from production, hurting their bottom line. Not only is the startup cost of a new vineyard unreasonable compared to many other crops, estimated in 2016 by Texas Tech University at over \$20,000 per acre in the first three years (Pate et al, 2016), but this also forces producer to forfeit any chance at achieving “old vine” status. “Old vine” is an arbitrary term used to market wines produced from more mature, long-standing grapevines. While any benefits of these wines are scientifically unsubstantiated, there is a perception amongst consumers that “old vine” wines produce superior, more complex wines than younger vine counterparts, and can be used as a tool to obtain premiums at market over otherwise indistinguishable products (Grigg, 2017). While grape growers are free to file the same crop insurance claims as other specialty crop producers when encountering dicamba injury in their fields, no dollar amount put towards yield loss or re-establishment costs can make up for lost years of productivity and the effort spent getting there.

Another issue with this is that young vineyards do not make money - for a moderate 20-acre vineyard, it takes about 13 years just to break-even (Olen and Skinkis, 2018). Simply put, vineyards cannot be replaced in the same manner that one could replace any planting of tomato or soy. If current dicamba usage patterns continue, affected grape producers will be forced to maintain dicamba-weakened vines potentially for decades after exposure, as starting anew even once every ten years would be economically infeasible.

Dicamba Symptomology in Grapevine. In susceptible plants, dicamba produces unmistakable upward cupping or epinasty in the leaves, as well as distortion of their stems (Egan et al, 2014). On grapevines in particular, dicamba injury appears as leaf cupping with restricted bands of marginal growth, but has also been correlated with the appearance of brown and yellow interveinal lesions, limited root structure development, primary bud necrosis, reduced leaf biomass indicating lower levels of photosynthesis, reduced berry weight, and uneven ripening of berries. Shoot tips may reveal a wilted appearing just one day after exposure. When shoot death occurs, the apex rarely resume growth after application. Trophic responses of grapevines to dicamba have also been seen as severe twisting and distortion of the petioles. Multiple exposure events can produce especially severe injury symptoms (Ball et al, 2004; Rossouw, 2018). Experimental phenotypes used to evaluate dicamba injury in the past have included general injury/death, leaf cupping severity, and reductions in shoot length. Leaf cupping is the most distinctive symptom of dicamba exposure in grapevines and has been shown to differ in severity between grapevine cultivars (Wolfe, 2013). Leaf cupping damage is manifest when leaf marginal meristems curl in on themselves, and is seen more commonly with upward cupping, but also can be visible as downward. The severity of cupping is also variable, as the curling can move inward on these meristems to the point where entire leaves have a bowl-like semblance (Wolfe, 2013).

Cases of Dicamba Drift Damage in Vineyards. For each of the documented cases of dicamba drift damage, there are likely countless more that go unreported. In many cases, reporting dicamba drift may prove more hassle than help, as state-run investigations into dicamba injury complaints only fine applicators for improper usage of dicamba, with no ability to compensate the victim (Unglesbee, 2018). Still, several cases of catastrophic vine loss to dicamba has been reported. In Pulaski county, Indiana, one grape farmer lost 810 established vines to an alleged dicamba injury from a neighboring field in the 2017 season (Binder, 2017). That same year, an Illinois farmer lost nearly half of his twelve acres of vines to dicamba drift just four days after his neighbor sprayed dicamba on resistant soybeans. After seeking guidance from one of his expert buyers – he was instructed to tear the vines out and start anew in the spring. Finally, after deciding to file for compensation from his crop insurance company, the claim was denied (Graef, 2017). With the ever-present threat of dicamba drift, growing grapes has become risky business in many parts of the US. The system of dealing with dicamba drift is currently too immature and ineffective to be relied upon. Another case, occurring in 2019, highlights that dicamba drift may be an inescapable foe for many grape growers, even when all the correct preventative measures have been utilized. Jennie Schmidt, a viticulturalist from Maryland, had her story detailed in DTN’s *progressive farmer*:

“[Schmidt’s] fields are registered with FieldWatch, she communicates annually with neighbors and actually took over weed control from the railway along the stretch of railroad that runs next to her vineyard to make sure herbicides stayed put... [This season,] Schmidt has watched extensive herbicide drift shrivel, pucker and stunt her vines, producing irregular grape clusters that taper into dead, burned tips, with small, inedible berries. Local experts believe her vines were hit by 2,4-D, dicamba, or both, up to three times this summer... While she is accustomed to some level of chemical drift most years, her vineyard usually grows out of it. This year, when it became clear that she would lose \$50,000 worth of contracts with local wineries, Schmidt reluctantly reported the injury to her state department of agriculture” (Unglesbee, 2019b).

Despite Schmidt's wholehearted effort to eliminate the threat of herbicide drift within her vineyard, drift persisted. Her story communicates not only the very real dangers of dicamba drift to specialty crop growers, but also the staunch opposition to government reporting and interference shared by many agriculturalists. While it is unclear the total economic impact of dicamba on the grape and wine industry, dicamba has demonstrated the capacity to destroy entire vineyards, and by extension, the livelihood of their owners. Growing grapes near dicamba-resistant row crops can cause serious issues: with such widespread and ubiquitous applications of this uncontrollable herbicide, it is nearly impossible to prevent drift injury from reaching ones' crops.

Economic Importance of Grape and Wine in Missouri. The grape and wine industries in the US are of immense value. In fact, the domestic revenue from wine in the US totaled \$42 billion in 2018, more than the value of soybeans harvested in 2016, priced at \$41 billion (American Soybean Association, 2017; BW166, 2018). Vineyards and wineries in Missouri are no exception to this: grapes and wines are a substantial contributor to the Missourian economy as well. Grape plantings take up 1,700 acres of cropland in Missouri, a humble figure compared to the almost 6 million acres of soybean in the state (USDA NASS, 2017; USDA NASS, 2019). However, this small acreage comprises over 130 wineries responsible for an estimated \$1.7 to \$3.2 billion in annual contributions to Missouri's economy when accounting for taxes, sales revenue, agritourism, and workers' wages (Frank, Rimerman and Co., 2015; Wine America, 2017). Major disruptions to vineyard management, such as could be caused by excessive dicamba vapor drift, would be disastrous to the feasibility and longevity of these vineyard operations. Currently, grape producers are taking precautions to avoid dicamba drift in their

vineyards, however it seems inevitable being interspersed among millions of acres of soybean. It is possible that dicamba drift injury will threaten Missouri grape growers for decades to come.

Possibility of Dicamba-Resistant Grapevine Cultivars. In the wake of this dicamba dilemma, planting dicamba-resistant or tolerant grapevine varieties would be an attractive option to many producers. Unfortunately, little research has been published on this subject, and as of 2019, no grapevine cultivar has been unanimously recognized as having such tolerance or resistance to dicamba drift. However, variation in the phenotypic responses between different grapevine varieties has been recorded. In one such experiment to look at the effect of dicamba in grapes, Ohio State University researchers tested five different varieties of grapevine to assess injury characteristics, performing a spray drift simulating assay of dicamba. Their findings reported that their European *Vitis vinifera* grapevine varieties showed greater injury symptoms than French-American hybrid varieties. It was also noted that these results of *vinifera* showing greater sensitivity than hybrids were shown consistently, with reports by Dami (2002) and Jiang et al (2010) (Wolfe, 2013). It is possible that such dicamba tolerance traits already exist in grapevines, and that they have simply not been discovered yet.

On GE Grapevine Varieties. Creating transgenic grapevines like the resistant soybean and cotton lines, which are able to detoxify dicamba, would be the ultimate solution for grape producers dealing with herbicide drift. Between the long generational time required for grape breeding, combined with an industry hesitant to adopt GE technology, grapes are not a likely candidate to become the next big commercial transgenic crop. When discussing the idea of genetically modified grapes cultivars with industry producers, there is a fear that such technology could lead to cancer and other health concerns (Cummins and Ho, n.d.). It seems many of the fears from GE grapes are rooted more in conjecture than scientific analysis. Even so, the

introgression of GE yeast itself in commercial winemaking has been met with a backlash (Pérez-Torrado et al, 2015). While there are definite possibilities of creating GE grapevines, the current market does not favor such changes, and is unlikely in the coming years.

Breeding Dicamba-Tolerant Grapevines. Without the feasibility of using of transgenic materials in grapevine, it may still be possible to create tolerant varieties using available *Vitis* germplasm, concurrent on identifying existing tolerance traits in grapevine cultivars already grown. While perception of genetically modified grapes may change in the future, current producers are sticking with traditionally bred varieties. If the viticulture community were to discover a single instance of dicamba tolerance in a commercially cultivated or wild variety of grapevine, it may then be possible to breed this variety with other popular vines, then selecting for the dicamba tolerance characteristic. Many disease resistance traits, particularly for downy mildew (*Plasmopara viticola*) and powdery mildew (*Erysiphe necator*), have been discovered in both cultivated and wild grapevines. Vines with these desirable traits are bred with more desirable, commercially successful varieties to produce the most profitable vines for plantings affected by disease (Miquel, 2018). Dicamba drift may be fought in vineyards in the same ways that fungal diseases have been, using resistant plant material to breed commercially viable genotypes to be used in areas at high risk of dicamba injury.

Study Overview

Experiments were performed to assess the severity of dicamba induced injury between two different existing grapevine cultivars: chambourcin (Ch) and cabernet sauvignon (CS). Two different types of experiments were designed to test chambourcin versus cabernet sauvignon for tolerance traits using dicamba drift assays. One experiment will investigate the phenotypic

response of young grapevines to spray drift simulating foliar applications of dicamba in the greenhouse; the other investigating the effect of dicamba vapor on young detached grapevine leaves of each cultivar. Both experiments attempt to determine specific dicamba concentrations, as well as days after treatment (DAT) that symptoms begin to appear, and when plants demonstrate maximal phenotypic damage.

Chambourcin and Cabernet Sauvignon. Grape varieties in this study were selected based on their potential value in future grape breeding efforts. The goal of such future breeding work would be to produce high-quality wine grapes which can tolerate unexpected dicamba drift events, that are also well suited to the unique growing conditions of the Midwest and Eastern United States. The French-American hybrid cultivar chambourcin was selected for this experiment as it has been purported to have a good level of tolerance to dicamba, according to a cultivar assessment compiled by Domoto, reported by Iowa State University (Smiley, 2016). Chambourcin also remains well suited to the climate in Missouri, making up greater than 10% of vineyard acres in the state, with over 80 different chambourcin wines released by Missouri wineries (Missouri Wines; Missouriwinecountry.com). Many who have tasted chambourcin wines will admit it is not their favorite, that it lacks the superior taste of the wines created from European grapes like cabernet sauvignon or merlot. The other selected cultivar for experimentation is cabernet sauvignon, a pure, European *Vitis vinifera* cultivar. Cabernet sauvignon is a traditional, premier red wine grape extensively used in commercial production. While not specifically mentioned in existing dicamba-related studies, cabernet sauvignon is presumed to have a greater susceptibility based on its *vinifera* genotype. Cabernet sauvignon remains difficult for Midwestern producers to maintain in the vineyard, due to disease pressure and lack of cold hardiness, and thus has room for many areas of genetic improvement. Cabernet

sauvignon is assumed to be susceptible to dicamba due to its genetic background, however no field studies have been published to confirm or deny this. Creating an interspecific hybrid breeding program between chambourcin and cabernet sauvignon could lead to the possibility of higher quality grapevines in the Midwest. If either of these cultivars demonstrate a tolerance to dicamba, that would simply become another trait to breed for, alongside disease resistance and cold hardiness.

With so many complimentary phenotypic characteristics between chambourcin and cabernet sauvignon, the potential for commercially successful crosses from these grape cultivars is high. An existing true hybrid population of 306 individuals planted in 2014, made from a cross of Ch (female parent) with CS (male parent), is under the management of Missouri State University in Mountain Grove, Missouri. This population was designed with the goal of combining the best traits from both parents in at least one individual vine, which can then be selected for later breeding or cultivar release.

Study Goal and Design. This study aims to discover whether chambourcin displays phenotypic tolerance to drift-simulating concentrations of dicamba when compared to cabernet sauvignon. Based on previous research of hybrid grapevine performance compared to *Vitis vinifera*, it is hypothesized that chambourcin vines will have less severe phenotypic injury than cabernet sauvignon, at all comparable rates and dates measured. This will be investigated by exposing grapevine cultivars to several, sub-lethal concentrations of dicamba, then evaluating the response of the vines over time. Protocols for each experiment are compiled based on existing research put forth by Scott Wolfe (2013), using phenotypes previously correlated with dicamba injury, including leaf cupping and changes in shoot length, as well as investigating the phenotypes of stem and petiole distortion, and leaf necrosis. These phenotypes will be used to

determine specific dicamba concentrations and DAT at which to show possible dicamba tolerances. Alongside testing chambourcin and cabernet sauvignon with one another, the protocols and methods of phenotypic observation themselves will be examined in terms of effectively correlating with dicamba drift. These evaluations will hopefully be used to guide future dicamba drift simulation experiments.

Spray Drift Simulation. Four related experiments were conducted to evaluate the phenotypic response of two different grapevine cultivars to drift-simulating foliar application rates of dicamba. These experiments were designed to investigate the timeframe for development of dicamba induced symptomology; herbicide concentrations at which injury phenotypes appear, or do not appear, in these grapevine cultivars, and if any significant level of tolerance exists in either cultivar relative to the other. Herbicide rates were designed to be sub-lethal and meant to simulate the effects of dicamba spray drift events. Previous spray drift assays of dicamba performed on grapes have used rates of 1/300, 1/100, and 1/30 of the label rate to produce sublethal effects (Wolfe, 2013). Depending on the applicator's boom height and gallon per acre (GPA) nozzle output however, the corresponding concentration of the dicamba necessary to apply this label rate can be drastically different. Under the most extreme on-label conditions, achieving the recommended label rate of 0.5lb ae per acre in a single pass could require tank concentrations of dicamba to be between 700 and 6,700 ppm ae, respectively. (TeeJet, 2017). Thus, the arbitrary value within this range of 6,000 ppm was set as the "typical label rate" to adapt these studies. Special care was also taken surrounding spray events to reduce the effect of dicamba from affecting plants within the experiment from drift, as well as nearby plants susceptible to drift. Therefore, dicamba sprays occurred in greenhouses, with quarantine periods no less than 24 hours between plants sprayed with different concentrations, to limit unintended

drift damage between treatment groups (Hartzler, 2017). While each experiment was designed following the same goals and generally the same protocol, slight changes in design, such as the rate of dicamba, between experimental designs were made continuously based on previous experiments' results. Different phenotypes were recorded between trials, including leaf cupping, stem and petiole distortion, and changes in shoot length. All experiments are referred to by the year the study was conducted, and then number based on chronological occurrence (ex: 2018, experiment two). These experiments involve young grapevines being sprayed with dicamba in a greenhouse assay to measure differential responses between cultivars.

Vapor Drift Simulation: Detached Leaf Assay. To investigate the effect of vapor drift on each cultivar, *in vitro* experiments were designed to predict dicamba resistance in vines using detached leaves. Four different experiments were performed with a goal of creating a replicable protocol that induces herbicidal damage in detached grape leaves through exposure to dicamba vapor. If successful, such a protocol could be used to investigate variation in phenotypic responses between individual plants without the need to expose entire plants to dicamba. Instead of propagating vines for the sole purpose of experimentation, which requires labor, time, resources, space, and capital, to test vines for dicamba susceptibility or tolerance, one could simply sample multiple leaves from individual plants for experimentation. While detached leaves do not contain the exact protein compositions seen *in vivo* and may not be a true indicator of how a live plant may react to stimulus, the validity of such tests to predict disease resistance has been made apparent previously, primarily in fungal disease assays. The aim of this study was to perform a protocol that could determine a range of dicamba concentrations for which grape leaves show chemical damage from dicamba exposure. The lack of information surrounding the effects of dicamba in detached leaves necessitated a *de novo* protocol. The major obstacle to this

study was the long period of time required for dicamba-induced leaf cupping to occur, combined with the short amount of time detached leaf tissue remains viable for experimentation. Trial and error was utilized surrounding experimental protocols in attempts to accelerate dicamba injury as well as to prolong the experimental life of detached leaves. Phenotypes of detached leaves are limited: contradictory information between the effects of auxin and dicamba on leaf senescence were confounding (Ball et al, 2004; Lem et al, 2010), and thus it has yet to be shown whether one can expect higher or lower levels of necrosis on leaves highly affected by dicamba. The length of each experiment was not pre-determined: instead, leaves were scored daily after dicamba exposure until plants were too far necrosed or had gone entirely limp. Due to lack of protocol surrounding this area of study, ad hoc changes were made between experimental design in different sets of detached leaf assays in attempts to elicit the distinctive leaf cupping phenotype produced by dicamba.

METHODS AND MATERIALS

Shared Methods and Materials

Study Site. All experiments were performed at Missouri's State Fruit Experiment Station, operated by Missouri State University in Mountain Grove, Missouri (9740 Red Spring Rd, Mountain Grove, MO 65711; Latitude 37° 09" Longitude 92° 16"), located in USDA hardiness zone 6b (USDA ARS, 2012). Spray drift simulation experiments were held onsite in custom greenhouses, while detached leaf assays occurred in the basement laboratory of Shepard Hall.

Herbicide Formulation. Vanquish (by Riverdale, Burr Ridge, Illinois), a DGA salt of 3,6 – dichloro-anisic acid, which is 38.5% dicamba acid equivalent (ae) or 4.0lb ae per gallon, was the only chemical utilized in any of the following experiments. Dicamba treatment solutions were diluted solely with diH₂O. Plants used in these experiments received no additional herbicidal applications.

Cultivar Selection. Chambourcin (Ch) and cabernet sauvignon (CS) were the only grapevine varieties sought to be tested on here.

Sources of Plant Propagation Material. Ch vines were obtained from cuttings in the Certified Virus-Free Vineyard (Foundations) at Missouri State University's Fruit Experiment Station (Mountain Grove, Missouri). CS vines originate from cuttings taken in the Genomics Vineyard of Missouri State University's Fruit Experiment Station. (Mountain Grove, Missouri).

Spray-Drift Simulating Experiments

Green cuttings. The first two out of the four spray drift simulating experiments were performed in late 2018 using green (softwood) cuttings of each cultivar as propagation material. Green cuttings were taken from clonal blocks of mother vines of Ch and CS in late Spring and early Summer (between June 7 and July 4). Cuttings were taken during early morning when vine water potential was highest. Partially lignified green shoots were selected and removed from the mother plant by clipping the vine at no further point basipetally than the internode where the cane loses its flexibility; were no longer easily bent between the thumb, index, and middle finger. Shoots were trimmed by removing all tendrils and odd numbered leaves (counting from the basal end). To prevent damage from water loss between the field and the propagation bed, this basal end of each detached cane was submerged in a bucket with approximately four inches of water and placed in the shade following removal and subsequent preparation. Two-node cuttings were created by clipping the shoot stalk of these prepared canes just above the nodes with remaining leaves. Large leaves were trimmed to approximately 1/3 of their original surface area to prevent excessive transpiration and potential desiccation of plant tissue prior to root establishment. Non-lignified portions at the top of canes (where shoot width was approximately half that of a pencil) were discarded and not used as propagation material. Shears were cleaned with bleach wipes between mother plants to lessen the risk of human-vectored disease within the mother plants as well as the cuttings. In a greenhouse, the basal ends of the prepared 2-node cuttings were dipped <1 cm in 1000ppm indole 3 butyric acid (IBA, Hormodin-1) powder to promote rooting, with excess powder tapped off. Cuttings were then gently inserted into nutrient sponge cutting media (Q plugs, iHort) and placed in Styrofoam holding trays under an intermittent mist system. Mist was set to spray between five and ten seconds every five minutes. Cuttings were labelled and numbered based on cultivar, mother plant, and date of cutting. Rooted cuttings were transplanted

after four to seven weeks under the mist system. The presence of roots producing from the bottom of the sponge media signaled sufficient growth for transplant. Plants were moved into pre-moistened sphagnum peat moss-based germination media in 40 oz. Styrofoam cups with drainage holes and moved into a large, general purpose, temperature-regulated greenhouse. Plants received water daily or as needed.

Hardwood Cuttings. The spray drift simulation experiments which took place in the 2019 growing season utilized vines produced from hardwood cutting propagative material. Hardwood cuttings of 3-4 nodes were taken from dormant, mature CS vines in December of 2018. These cuttings were wrapped in damp newspaper, placed within an unsealed plastic bag, and then refrigerated. These cuttings were taken earlier than Ch for fear that frigid temperatures would knock out the viable propagation material on the limited number of CS mother plants available in Mountain Grove. A month later, on January 10, the first hardwood cuttings of Ch were obtained. On the same day, the initial cabernet sauvignon cuttings were removed from refrigeration. Tied bundles of 10 to 25 cuttings of each cultivar had their basal ends dipped into 0.1% IAA powder (Hormodin 1), and then placed 10 to 15 cm deep within a rooting bed filled with moistened peat-based germination media set at 75 degrees Fahrenheit, however actual temperatures fluctuated between 78 and 85 degrees. The rooting bed, placed inside a cold, dark cellar, received water as necessary: typically, once or twice a week. After 5 weeks in this rooting bed, when both cultivars exceeded 60% rooting, rooted cuttings were transplanted into peat-based germination mix in four-inch plastic nursery pots, then moved into the same large, temperature greenhouse for budbreak and shoot formation that was also used maturation for green cutting transplants. The rooting bed and rooted cuttings are shown in figure 1. Plants were numbered and labelled with information including cultivar, mother plant, and date of cutting.

These plants were used in the first of the two 2019 spray drift simulations. A second batch of hardwood cuttings of each cultivar was taken throughout the month of February and underwent the same rooting procedure as the hardwood cuttings listed above. The vines produced from this second batch of hardwood cuttings were used in the second spray drift simulation of 2019. The reason for staggering the propagation of these vines was to remove the variation of plant size and stage of growth between the two 2019 spray drift experiments.



Figure 1. Hardwood cuttings that have taken root (left) after several weeks of root induction in the rooting bed (right).

Herbicide Formulation. Vanquish (by Riverdale, Burr Ridge, Illinois), a DGA salt of 3,6 – dichloro-anisic acid which is 38.5% dicamba acid equivalent (ae), or 4.0lb ae per gallon, was the only chemical utilized in any of the following experiments. Dicamba treatment solutions were diluted with diH₂O and were not mixed with any additional surfactants or adjuvants in any of the experiments described below.

Spray Equipment. Herbicide applications were carried out using simple, hand-held trigger pump bottle sprayers with mist spray nozzles. This style of sprayer was selected for its consistent volume output and spray pattern, allowing for individual “doses” of herbicide to be reliably allocated to individual plants across the entire tested population. Other considerations for

using this style of sprayer are its low relative cost, and semi-disposable nature. Each treatment level of dicamba received its own sprayer bottle to limit the effects of residual solution lingering between treatments. Each years' treatments were performed using different brands of trigger spray bottles. The units used in 2018's experiments (Might Gadget R, 52151644) had an average output near 1.0 mL per pump, and 2019's sprayers (Housables, 24oz.) averaged just above 1.2mL per pump. Little variation between sprayers of the same model was recorded (data not shown). All sprays were conducted with sprayer nozzles held exactly 30 cm from the canopy of the target vine, with the spray occurring parallel with the ground.

Spray and Post-Spray Environment. Simple tent-style greenhouses (BenefitUSA GH055, 10'x7'x6'; Outsunny, 12'x10'x7') were used to facilitate sprays. Each tent was slightly modified by sealing ventilation ports to reduce indoor-outdoor gas exchange. To counter subsequent heat buildup in this environment, windowsill air conditioning units (General Electric AET05LY, 5000 btu) were installed in each greenhouse. These units ran on outlet timers set to kick on from 9am to 5pm and were only used when outside temperatures exceeded an average of 70 degrees Fahrenheit. Inside these greenhouses, benches were set up approximately 45cm off the ground to hold plants. Plants received water daily or as needed. Shade cloth was used to shield the larger Outsunny greenhouse during peak Summer heat. At canopy height, daytime temperatures in the Summer were never recorded above 105 degrees Fahrenheit.

2018 Spray Drift Experiments. Ch and CS vines were sorted into eight groups each, split into two sets of four treatment groups. The first set of plants corresponds to the first 2018 spray drift simulation, and the second set for the second 2018 spray drift simulation. For each experiment, there were four different groupings per cultivar, corresponding to a control group and three different treatment groups for different dicamba spray rates. Prior to group selection,

vines were scored on an ordinal scale one-to-five on quantity of new growth (one: no new growth; five: excessive shoot proliferation). Plants with no new growth were removed from the selection pool, and all other plants sorted from high scores to lower scores. For each cultivar, beginning at the highest rated plants, strata of eight individuals were sorted randomly into one of the eight groupings per cultivar using random number generation. For Ch groups, n=14 or n=15. For CS, n=13 or n=14. This and all other spray drift simulation experiments' variations are summarized in table 1.

Table 1. Summary of spray drift simulation experiments, detailing population sizes, volume of dicamba sprayed on plants, dicamba concentrations used for treatment groups, phenotypes assessed, and important dates for each experiment.

| Experiment | 2018, 1 | 2018, 2 | 2019, 1 | 2019, 2 |
|-------------------------|----------------------------|----------------------------|------------------------------|------------------------------|
| Plants of CS | 13-14 | 13-14 | 50 | 25 |
| Plants of Ch | 14-15 | 14-15 | 17 | 25 |
| Spray volume (mL) | 1.0 | 1.0 | 2 x 1.2 | 2 x 1.2 |
| Dicamba rates used, Ppm | 0,20,60,200 | 0,20,60,200 | 0,15,30,60,120 | 0,15,30,60 |
| Date of Cuttings | 6/7-7/4 | 6/7-7/4 | 12/10 (CS), 1/10 (Ch) | 2/7-2/14 |
| Date of Transplant | 7/23-7/30 | 7/23-7/30 | 2/12 (CS), 2/26 (Ch) | 3/26 |
| Treatment date | 9/5-9/7 | 9/19-9/21 | 4/10 (CS), 5/6 (Ch) | 5/28 |
| Duration of study | 44 Days | 30 Days | 14 Days | 70 Days |
| Phenotypes Assessed | Leaf Cupping Distortion | Leaf Cupping Distortion | Leaf Cupping Shoot Length | Leaf Cupping Shoot Length |

2019 Spray Drift Experiments. For the first spray drift simulation of 2019, plant group selection began at two months after initial transfer of rooted cuttings into the greenhouse for shoot development. Plants with active growth were recorded, and randomly grouped into six different treatment (one control; five dicamba concentrations) for CS, and five groups for Ch

(one control; four dicamba concentrations). The propagates taken from CS in December were premature when compared to the Ch cuttings taken almost a month later, despite being placed in the rooting bed on the same date. Thus, in order to expose each vine to dicamba at a similar stage of growth, CS vines were treated with dicamba several weeks before their Ch counterparts. A substantially larger number of vines was available for CS treatment groups (n=50) than in Ch treatment groups (n=17). In 2019's second spray drift simulation, CS and Ch vines with active growth were sorted into four distinct groups using a random number generator (one control; three dicamba treatment concentrations). All treatment groups: n=25.

Spray Logistics. Spray logistics and procedures varied year to year and even trial to trial as protocols were tweaked. Important aspects of the design, along with similarities and differences between runs are noted here.

2018 Spray Drift Experiments. All herbicide applications occurred before noon and were confined to a single greenhouse. Spray applications took place over a three-day period. The three greenhouses used in these experiments will hereupon be referred to as GH1, GH2, and GH3. GH1 and GH2 are the same size and located behind the northern wall of a storage structure. GH3 is bigger than the other two greenhouses. On day one, control vines of both cultivars were placed in GH3 and were treated with a foliar application of water. The same day, Ch and CS vines from the first (lowest rate) dicamba-treatment group were placed in GH1 and treated with dicamba. Following herbicide application, plants were quarantined within GH1 for a 24-hour period after application to lessen the effect of dicamba vapor affecting the control group or other treatment groups. On day two, plants from GH1 were moved into GH2. The next highest treatment level of dicamba was brought into GH1, and sprayed with dicamba. On day three, the plants from GH1 were again moved to GH2, and the third (highest concentration) treatment group were moved

into GH1 and sprayed with dicamba. One week after the initial spray, all plants were relocated to GH3 and maintained here for the remainder of the experiment. Both 2018 spray drift experiments were performed in this manner.

2019 Spray Drift Experiments. The following paragraphs detail the different spray logistics utilized during the 2019 spray drift experiments. While the trials remained generally the same, the similarities and differences are noted below.

First Experiment 2019. As in the 2018 runs, all herbicide applications occurred in one greenhouse. However, all sprays occurred in one day, and only GH1 and GH3 were utilized. For this experiment, all plant groups were placed in modified lugs that could be covered. Each container had raised poles in each corner which extended above the vines' canopies. Sprays occurred solely in GH1, and began with the control, followed by subsequent treatment groups in the order of increasing concentrations of dicamba. For each treatment group, plant lugs were placed after spray exposure into large, polyethylene containers resembling oversized garbage bags, and sealed with duct tape to prevent outside air exchange and thus vapor exchange between treatment levels. These sealed containers were then moved to GH3. Upon carrying out this procedure for all treatment groups, plants were left sealed in their containers for 24 hours following the final spray. At 24 hours, all plants were removed from their sealed containers and left in GH3 for the remainder of the experiment.

Second Experiment 2019. Four different greenhouses were utilized for this experiment's sprays. All sprays occurred on the same date and had each of the four treatment groups occupying their own greenhouse. The control group was treated in the large, general greenhouse mentioned in the green and hardwood cutting protocol. The lowest rate of dicamba was sprayed in GH3, the next highest in GH1, and the final concentration in GH2. One week after spray, all

dicamba-treated vines were moved to GH3, while the control plants were maintained away from possible dicamba vapor in the general population greenhouse. These placements were maintained for the entirety of the experiment.

Herbicide Rates. The 2018 spray drift simulation rates are based on these spray rates from Scott Wolfe's 2013 thesis investigating low rates of dicamba and other herbicides on grapevine (Wolfe, 2013). For both 2018 spray drift simulation assays, an assumed a spray rate of 10 gallons per acre are used for application with a corresponding concentration of 6,000 ppm ae of dicamba for a 0.5lb ae per acre spray rate. 1/300, 1/100, and 1/30 of this concentration are 20, 60, and 200 ppm, respectively. Thus, the concentrations spray on different treatment groups were 0, 20, 60, and 200 ppm dicamba ae of Vanquish. Undiluted Vanquish has a dicamba ppm ae of 400,000.

In the 2019 experiments, previous rates were modified based on assessments of 2018 phenotypic data. The concentrations used in the different treatments of the first 2019 spray drift experiment were 0, 15, 30, 60, and 120 ppm dicamba ae of Vanquish for each cultivar. In the second 2019 spray drift assay, spray concentrations were further tweaked from results of the previous 2019 experiment. Only four treatment groups were used, with rates of 0, 15, 30, and 60 ppm dicamba ae.

Spray Procedure. Plants in 2018 were sprayed with no particular target toward their canopy, with the goal to hit the most surface area over the plant as possible from one single spray trigger pump. In 2019 experiments, instead of indiscriminately spraying the whole canopy, individual vine shoots were tagged with plastic coated twist ties: these shoots would then become the specific target of a spray application. 2019 spray procedure also differs from the 2018

procedure in that two trigger pumps of solution were applied to the target shoot at 30 cm from the canopy. Typically, the top and then the bottom of the target shoot.

Phenotyping. Phenotypic measurements were taken just before the experimental treatment, as well as in weekly intervals after treatment, except for shoot length in 2019's second experiment. Scoring was completed in the morning hours to reduce the influence that time of day may have had on phenotypes. 2018 scores were reported by one individual; 2019 scores were recorded by two scorers.

Leaf cupping. In 2018, leaf cupping severity measurements were focused on scoring the healthiest leaf of each individual plant, recorded as a value along on a one-to-five visual scale, with increments every one-half a point. A score of one corresponds to a flat, pristine leaf, while five denotes a severely cupped leaf. Figure 2 shows a visual representation of this scale. In 2019, leaf cupping denotes an average in leaf cupping across all leaves on the targeted shoot and is scored on the same one-to-five scale as 2018.



Figure 2. Photographs visually demonstrating the one-to-five leaf cupping scale, with one at the left, and five at the right.

Distortion. To determine the severity of each plant response, evaluators observed major twisting of vine stems and gave scores on the average of a twisting and distortion over the entire plant. Scores were given on a one-to-five visual scale, with possible increments of one-half. A score of one denotes a healthy, undistorted plant, and five denotes a severely distorted plant.

Figure 3 visually describes the phenotypic distortion ratings in response to simulated herbicide drift. Only 2018 experiments were evaluated using this metric.

Shoot Length. Shoot length was determined on the target shoot by measuring from the base of the shoot, where the axillary bud first burst, to the shoot apex. Scores were measured just prior to spray application and were collected again at later time points after dicamba exposure. Shoot lengths were only recorded in 2019 experiments.



Figure 3. Stem and petiole distortion photographs visually demonstrating one-to-five rating, 1 at left, 5 at right.

Data Reporting. Trends in the development of leaf cupping and distortion of stem and petiole in dicamba exposed grapevines are shown on a scatterplot. These charts track the distribution of phenotypic scores in each dicamba concentration over the entirety of the experiments. Two charts will be given for each cultivar for each experiment and phenotype reported. These charts are redundant in that they have the same points plotted, however one chart will be used to disseminate information on the frequency distribution of scores under different conditions and timepoints. Large translucent bands around points represent higher frequencies of individuals with that score. The other chart will show the same points as the first one and will display linear or polynomial regression lines for each dicamba treatment level over the course of the experiment.

Shoot lengths were reported as group means at different timepoints and for different treatments of each cultivar. Using SAS (version 9.4) for Windows, these group means were

compared using Tukey's Honest Significant Difference test. Significance between group means was determined with an alpha level of 0.05.

Detached Leaf Assay Experiments

Herbicide Rates. Dicamba concentrations were variable between the different experiments. Detached leaf experiments 1 and 2 examined the vapor drift effect of dicamba concentrations at 0, 15, 30, 60, and 120 ppm ae of dicamba. Experiments 3 and 4 examined the effect of 0, 15, 30, 60, 120, and 240 ppm ae of dicamba. Experimental design variables such as concentration and other experimental factors which differed between experiments are summarized below in table 2.

Table 2. Differences in detached leaf assay experimental designs from experiment 1 to 4.

| | Experiment 1 | Experiment 2 | Experiment 3 | Experiment 4 |
|-------------------------|--------------------|--------------------|-------------------------|-------------------------|
| Dicamba rates used, ppm | 0, 15, 30, 60, 120 | 0, 15, 30, 60, 120 | 0, 15, 30, 60, 120, 240 | 0, 15, 30, 60, 120, 240 |
| Dates conducted | June 11-15 | June 17-21 | June 26 | July 1-6 |
| Total leaves tested | 80 | 60 | 72 | 72 |
| Leaves/conc. | 16 | 12 | 12 | 12 |
| 24 Hr. Post-treatment | High temp | Room temp | High temp | Room temp |
| Petiole treatment | Removed | 2% sucrose | 2% sucrose | Removed |

Leaf Selection and Preparation. Translucent, undistorted grapevine leaf samples (approximately the third full leaf from shoot tip) were taken from mature plants of each cultivars for testing with full petioles intact. Leaf sampling occurred during early morning when plant foliar tissue is at its highest water potential status. Leaves which already appeared distorted in the field were avoided for sampling. Leaves were immediately transported from the field to the lab

for processing. In lab, leaves underwent surface sterilization in a 10% bleach solution for 30 seconds and were subsequently rinsed in several successive diH₂O baths to remove bleach residue. Petioles were removed entirely in experiment 1 and 4 subsequent to surface sterilization. Conversely, experiments 2 and 3 had just the tips of their petioles removed after rinsing, with the remainder of the petiole then inserted into sterilized cotton balls soaked in a 2% sucrose solution, which has been shown to delay senescence in detached leaves (Wolff and Price, 1960). Plants were kept within a sterilized laboratory hood with positive pressure in the presence of a lit Bunsen burner following surface sterilization to prevent the interference of pathogens in the development of herbicidal injury or senescence in the detached leaves.

Preparation of Vapor Chambers. 20mL aliquots of different dicamba concentrations were applied to autoclaved paper towels at the bottom of clear polyethylene containers approximately 8"x12"x3" in size. Each container held four leaves, two of each cultivar. An image of this set-up from experiment 1 is shown below in figure 4. In all experiments, leaves were placed on thin plastic elevated platforms to separate the leaf from the paper towel. This was done by utilizing petri dishes to elevate the leaves, or the plastic spacer seen in figure 4. These dividers effectively occluded the plant tissue from direct contact with the herbicide solution. In experiment 4, autoclaved filter paper or paper towels with 1mL of diH₂O were placed underneath each leaf sample. Lids were snapped onto containers in the hood, however the design of the containers prevented them from being completely airtight.

Post Exposure maintenance. Experiments 1 and 3 received incubation in a greenhouse with elevated temperatures (up to 95 degrees Fahrenheit) and in indirect sunlight for 24 hours after exposure to increase vapor levels within containers. Experiments 2 and 4 had their containers left at room temperature under 24 hour fluorescent lighting post treatment. After this

24 hour period, all experiments remained in an active laboratory setting at room temperature, and were neither supplemented with additional lighting, nor restrict from it. Each container was briefly opened once per day after initiation of the experiment to record and photographically document phenotype development to accurately phenotype detached leaves.

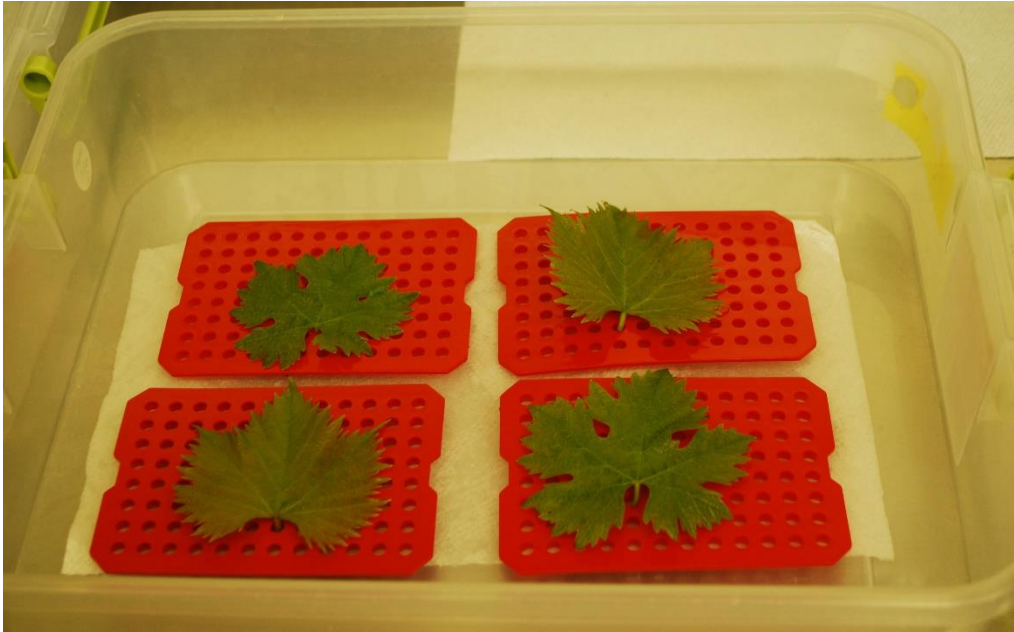


Figure 4. Image of detached leaf assay setup of experiment 1

Duration of study. The duration of each experiment was highly dependent upon the experimental conditions other than variable dicamba rates. No distinct stopping point for any detached leaf assays had been premeditated prior to experimentation. Leaves which had lost turgor pressure and gone limp, as well as brown, wilted leaves were assumed to no longer be actively growing, and thus would demonstrate no further development of visually apparent effects from dicamba exposure. Duration of each study is listed in table 2, ranging from 1 day of scoring to 6.

Phenotyping. Two different phenotypes were used to document dicamba injury and leaf health during the detached leaf assay. These characteristics and the means by which they were measured are offered below.

Leaf Cupping. Leaf phenotypes were scored on the same one-to-five scale with one-half point increments used in the spray drift simulating assay of dicamba mentioned previously, as well as shown in figure 2. Individual detached leaves were scored in this manner pre-exposure and daily thereafter. This phenotyping procedure involved briefly opening the container daily to get an unobstructed view of each leaf for more accurate phenotyping, as well as for better photographic documentation of phenotype development over the course of each experiment.

Leaf necrosis. Necrosis of detached leaves were scored *ad hoc* utilizing the photographs taken on the last day, 4 DAT, for experiment 1. Only experiment 1 necrosis was scored and analyzed. Leaf necrosis of detached leaves, while not previously confirmed to be affected by dicamba, has been shown to be affected indirectly by the effects of auxin, and has the potential to be used in future dicamba-tolerance phenotyping procedures. Necrosis of the leaves were scored on a one-to-five scale with increments of one half, with one representing completely green leaves, and five corresponding to leaves which completely lack chlorophyll and generally appear brown and shriveled. Figure 5 represents this scale visually.



Figure 5. A visual representation of the necrotic injury on grape leaves as a one to five scale, shown left to right.

Data Reporting. Trends in the development of leaf cupping and necrosis in dicamba exposed leaves are reported by scatterplot. These charts demonstrate the distribution of phenotypic scores among individuals of each dicamba concentration used, given over the timespan of the experiment. Separate charts are shown for Ch and CS. Two nearly identical charts will be given for each cultivar for each experiment and phenotype reported. These charts will be placed above and below each other and represent the exact same data. However, the upper charts will be used to disseminate information on the frequency distribution of scores of any treatment level at any given time point: with larger bands around points representing higher frequencies of individuals with that score, relative to the other individuals' scores. The below chart will show the same plot points as the first one, however instead of alluding to frequency distribution, will display linear regression lines for each dicamba treatment level over the course of the experiment. This is the same method used in the spray drift simulation to represent data.

RESULTS AND DISCUSSION

Spray Drift Simulations of Dicamba

2018: Experiment 1. Leaf cupping phenotypic scores are shown in scatterplots compiled in figure 6. At 0 DAT, Ch already demonstrates a high amount of natural leaf cupping, in sharp contrast to CS at the same time point. After the first week post treatment, treatment groups from each cultivar are already showing elevated levels of leaf cupping compared to their controls. Ch seems to have the largest disparity in leaf cupping scores between the highest concentrations of dicamba and the control near 7 DAT. At 14 DAT and thereafter, virtually no differences existed between the severity of leaf cupping in treated and non-treated Ch vines. The same cannot be said for CS. CS treatment groups saw a similar spike as Ch above their control in leaf cupping at 7 DAT, but these treatment groups would remain with a higher frequency of cupping for the remainder of the experiment, despite seeing decreases in treatment groups' leaf cupping after several weeks. Looking at the trendlines in the bottom scatterplots, Ch leaf cupping is relatively unresponsive to the additions of dicamba in treatment groups. All Ch groups, including the control, increased in leaf cupping at approximately the same rate as their trendlines run closely together, nearly parallel. The chart and trendlines for CS indicate this cultivar has a much stronger response to dicamba exposure, as higher treatment levels correlate to higher incidence of leaf cupping. The space between the trendlines for the control and different treatment levels of dicamba are much greater for CS than Ch. In this experiment, leaf cupping injury peaks around 14 to 21 DAT for nearly all treatment levels in each cultivar, with damage seeming to subside, and then plateau for nearly every group.

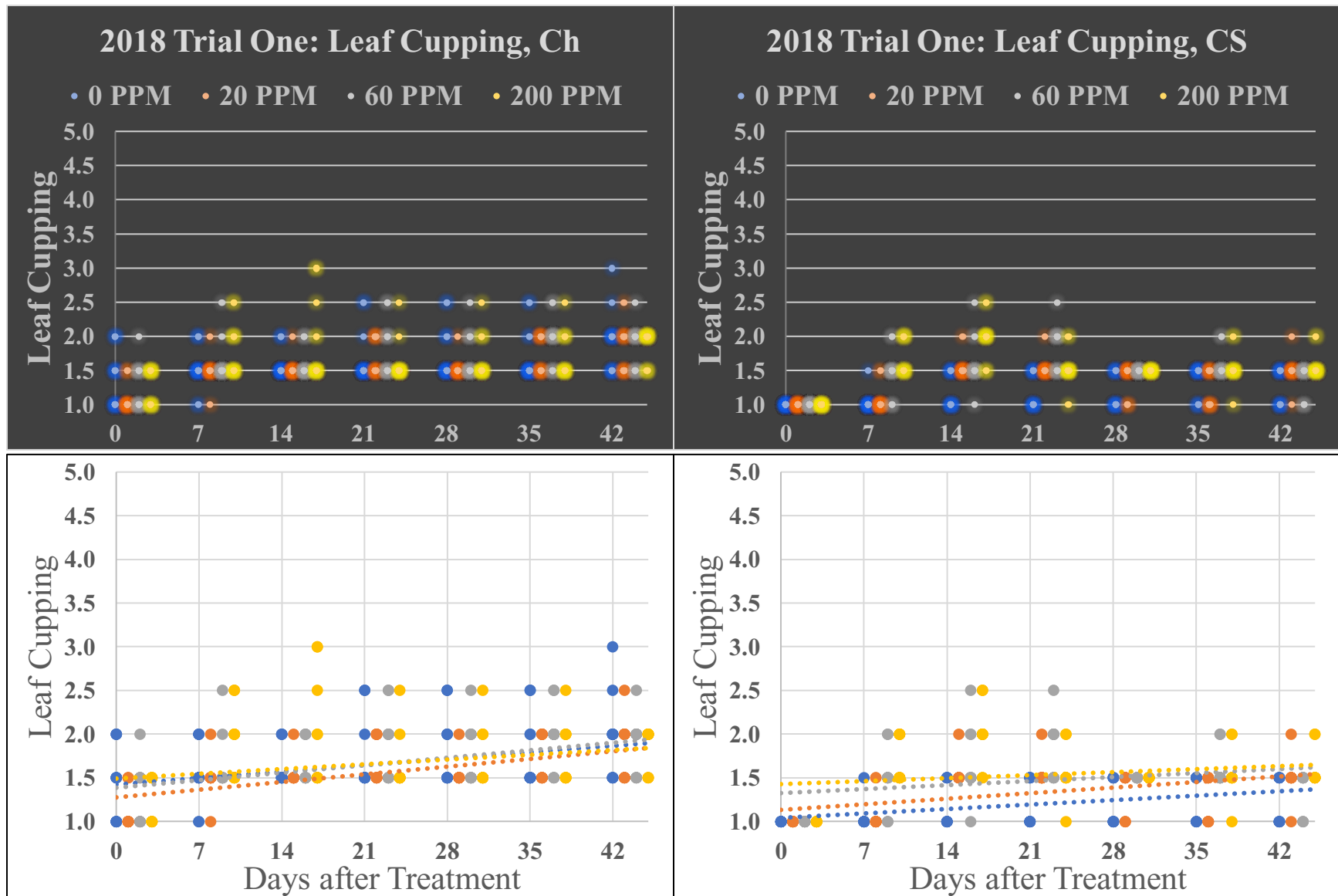


Figure 6. 2018 trial one leaf cupping score distribution and trends.

Observations from distortion analysis reveal similar results as the leaf cupping. These observations are reported in figure 7 below. In this figure, phenotypic differences between the control group and 200 ppm dicamba treated vines are seen in both cultivars at one-week post treatment. At 7 DAT under this highest concentration, CS tested vines all scored above any distortion injury rating given to its control group, where only a handful of Ch vines achieved this compared to their own control group. The lower treatment levels of CS at 7 DAT show slightly elevated levels of distortion when compared to its control, where comparable Ch plants were indistinguishable from untreated ones. Distortion ratings increased slowly in all experimental groups from 7 DAT to the conclusion of the experiment, as seen by the distribution of plant scores over time, as well as the linear regression lines given below in figure 7. For Ch vines below 200 ppm, there seems to be no discernable differences in the level of distortion, save for a few exceptions beginning at 21 DAT. The trendlines for the 0, 20, and 60 ppm Ch treated vines are nearly identical. At 200 ppm, only a few Ch plants showed high levels of stem and petiole distortion. These distortion levels, and the disparity between the control and this group increased steadily until 21 DAT, but then stayed relatively constant in subsequent recordings.

In contrast to the Ch scorings, the effect of dicamba on distortion can be seen at the first recording, 7 DAT, from even all treatment groups from 20 to 200 ppm. While differences between the 20 and 60 ppm groups are miniscule, they maintain a small, but consistently higher distortion score than the control vines for the entire experiment. These differences became more pronounced over time, as the 20 and 60 ppm groups continue to increase in distortion, with no indication of slowing down, up to 42 DAT. Rates of 200 ppm showed a quick and severe development of distortion characteristics for CS vines, substantially more so than any of the controls tested. This treatment group's distortion ratings only increased until 14 DAT, with

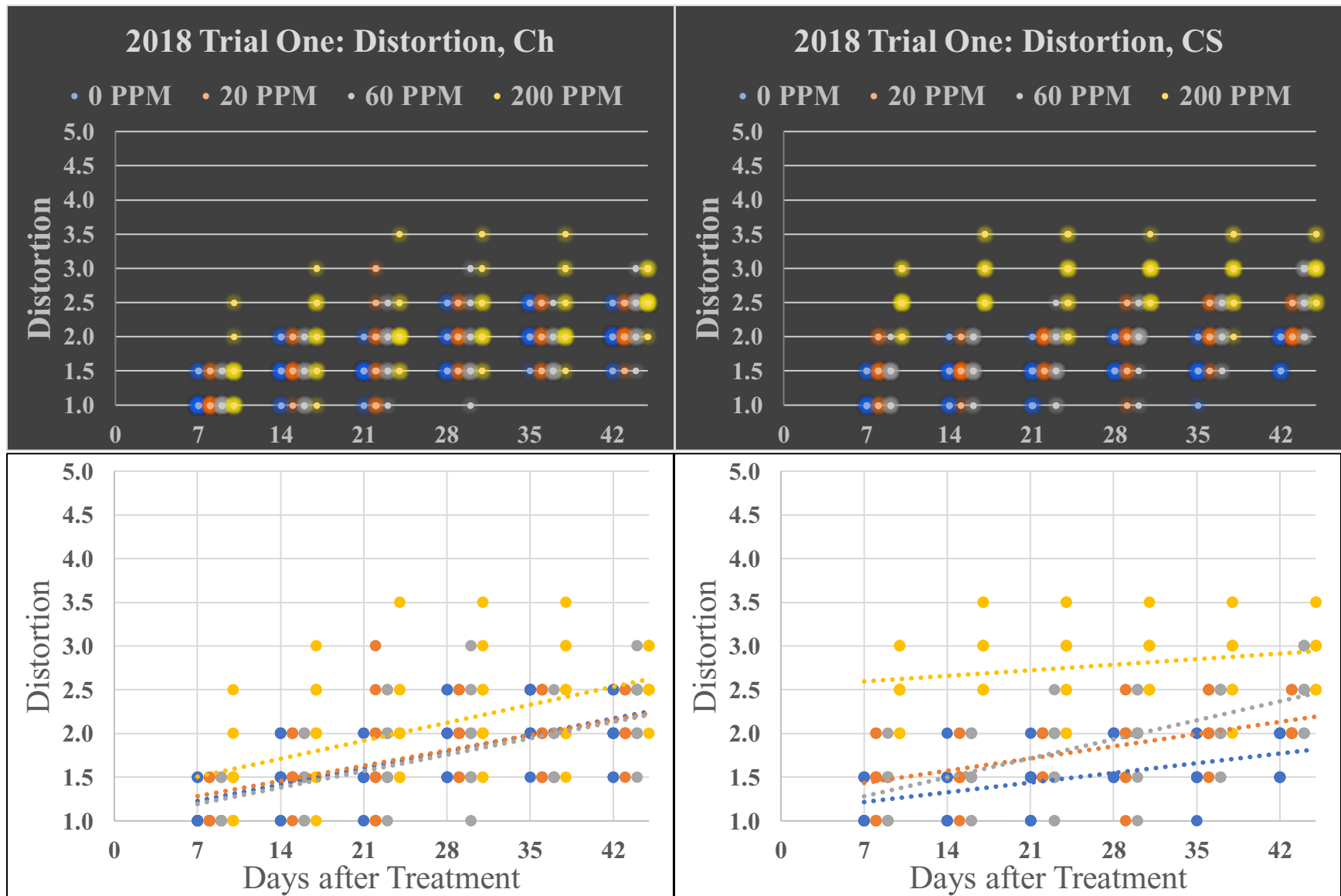


Figure 7. 2018 trial one distortion score distribution and trends.

the phenotype somewhat subsiding in later documentation.

Maximal differences between the control vines in each cultivar were seen in the 200 ppm treatment level. For Ch, this difference is small, and increases slightly over the course of the experiment. For CS, maximal differences between the highest treatment group and the control were seen at 7 and 14 DAT: large differences continued to 42 DAT, but were less severe as the control vines continued to develop a distortion phenotype when the highest exposure group had plateaued.

2018: Experiment Two. The distribution of leaf cupping scores in this second spray drift experiment are given in figure 8. Ch vines at all treatment levels are initially seen to have higher levels of leaf cupping than CS. That being said, leaf cupping ratings differ little between treatment levels in this experiment. Slight increases in leaf cupping scores are noticed across the board from 0 to 7 DAT, with increases across all experimental units, including the control. Over the course of the experiment, even the highest dicamba rates utilized saw little response from these vines, in both cultivars. Leaf cupping scores between groups of the same cultivar are largely indistinguishable. The greatest disparity between the control and any treatment group occurs at 28 DAT for Ch's 200 ppm group. The leaf cupping score of this group trends slightly higher than the other Ch groups, as it can be seen to have a trendline with a greater slope that intersects the other groups' trendlines. At 28 DAT, CS vines seem unaffected by dicamba at all treatment levels when using leaf cupping as a measure of dicamba injury. While all Ch vines steadily increase in leaf cupping up to 28 DAT, CS were most severely damaged at 7 DAT, and seemingly recovered in following weeks, as less severe leaf cupping ratings were recorded. Distortion of stems and petioles in 2018's second spray drift simulation experiment are reported

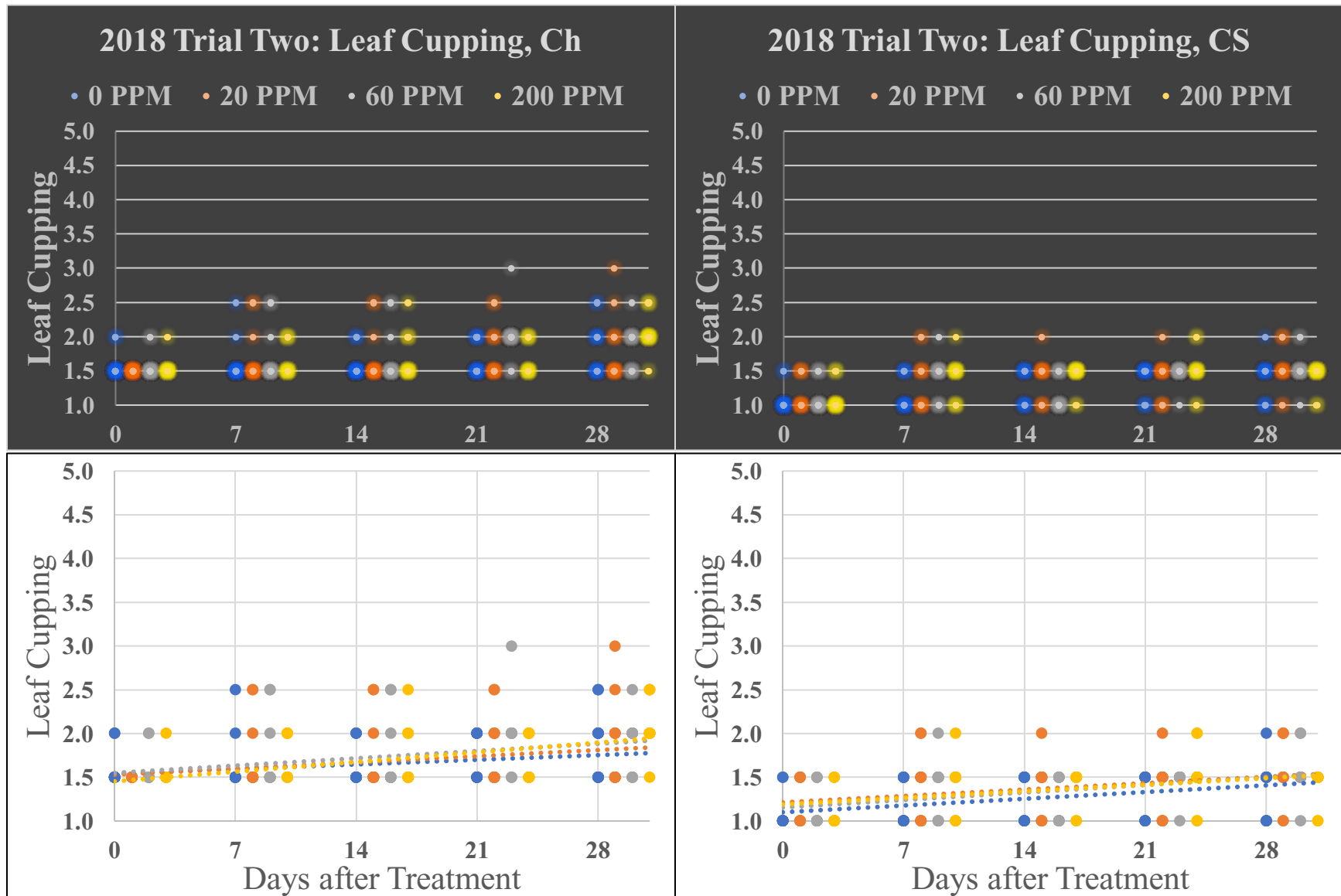


Figure 8. 2018 trial two leaf cupping score distribution and trends.

in figure 9. Values for both cultivars start out almost identically at 0 DAT, however disparities between cultivar responses to dicamba become evident at 7 DAT. Dicamba exposed vines of each cultivar demonstrate an elevated level of distortion from the control at one-week post application. This increase is subtler for Ch vines, while it is much more of a stark spike in distortion ratings for CS vines, especially at the 200 ppm level. While greater differences exist between the control group and treatment groups of CS than Ch, the trendlines reveal that these differences start early on and stay relatively constant over the course of the experiment, and even decrease as control vines continue to develop distortion symptoms while that of treated vines stagnates between 21 and 28 DAT. For Ch, 200 ppm distortion injury continues to increase, on average, to 28 DAT, and only shows maximum differences from control vines during the last recorded observation on this day. Development of distortion phenotypes plateaued for CS after 21 DAT in its 200 ppm groups. While there is a slight effect on CS vines after exposure to 20 ppm sprays of dicamba, it is apparent that higher levels of distortion are detectable in higher concentrations. Ch trendlines similarly show that dicamba rates of 20 ppm are insufficient to cause detectable distortion phenotypes. Looking at both cultivars, trendlines for scores of each treatments' individuals demonstrate that the control and 20 ppm treatment levels follow similar phenotypic tendencies, while 60 and 200 ppm levels are more closely lumped together.

While this experiment was planned to run out for a longer duration, it was cut short by the changing of the seasons and the accompanying natural senescence and decay of green tissue. Ch leaves first developed a red hue, beginning in interveinal regions before spreading throughout the whole leaf. This phenotype affected all treatment levels evenly, but this phenomenon was substantially less pronounced in CS vines. No differences in this phenotype between the

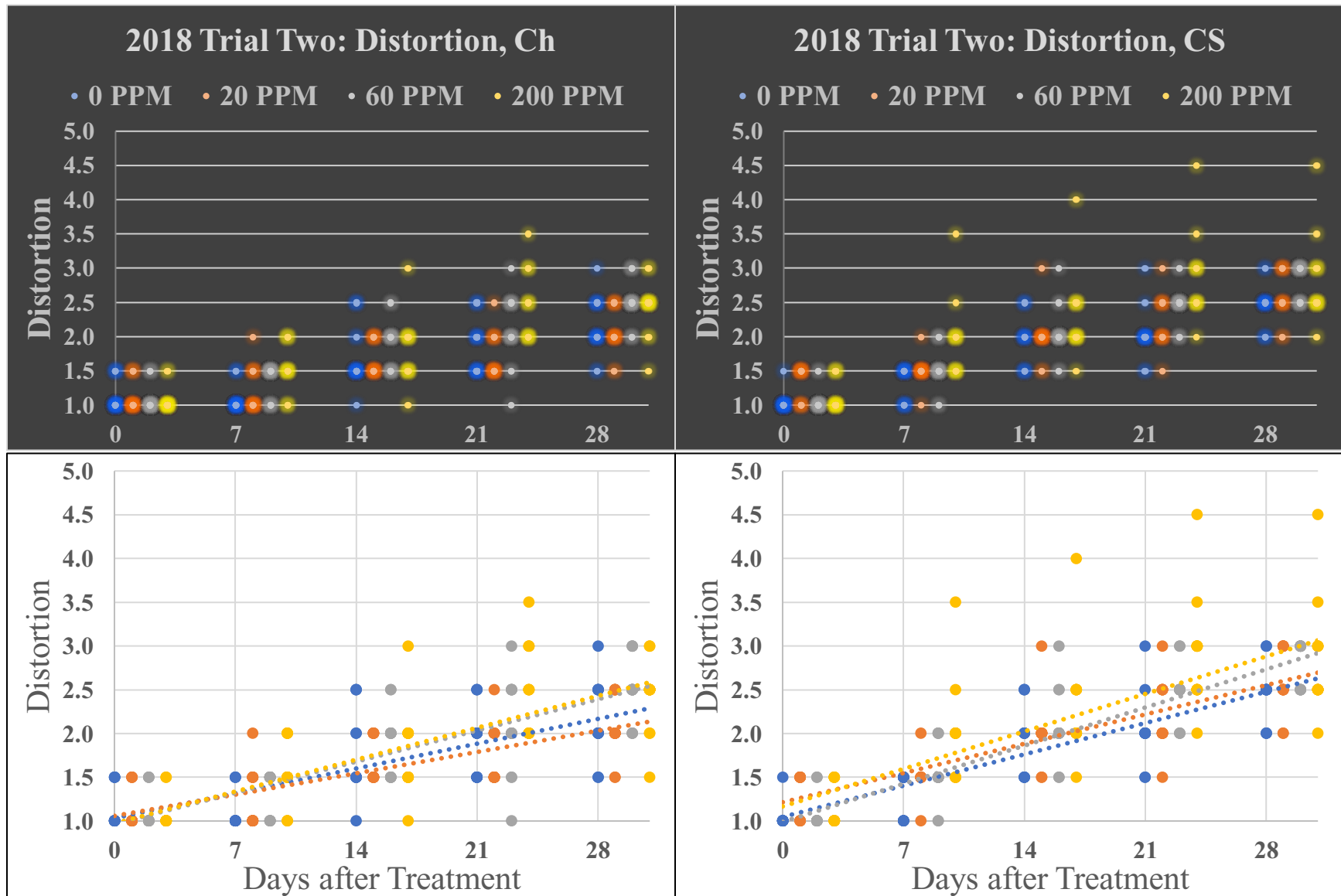


Figure 9. 2018 trial two distortion scores distribution and trends.

treatment levels were noted for CS either. Images of plants from the 2018 experiments, on the last recorded day of the experiment, October 19th, 2018, document the senescence phenotype of these vines. These images are shown in figure 10 below. The crates with predominantly red-leaved vines can be identified as Ch, and predominantly green as CS.



Figure 10. Photos of mixed Ch and CS (left), and individual Ch plants (right).

2019: Experiment One. Leaf cupping phenotypic distribution results are represented in figure 11 on the next page. Both cultivars demonstrate sensitivity to all treatment levels of dicamba, with phenotypic development of leaf cupping above the control visible at rates as low as 15 ppm after one-week post exposure. In both cultivars, increasing rates of dicamba correlate well with increasing leaf cupping scores. For Ch, 15 and 30 ppm demonstrate marginally worse leaf cupping than the control, while the differences seen in the 60 and 120 ppm treatment levels are much greater. In contrast, 15 and 30 ppm treatments on CS vines led to marked increases in leaf cupping over control group scores. Each successive increase in treatment level leads to more

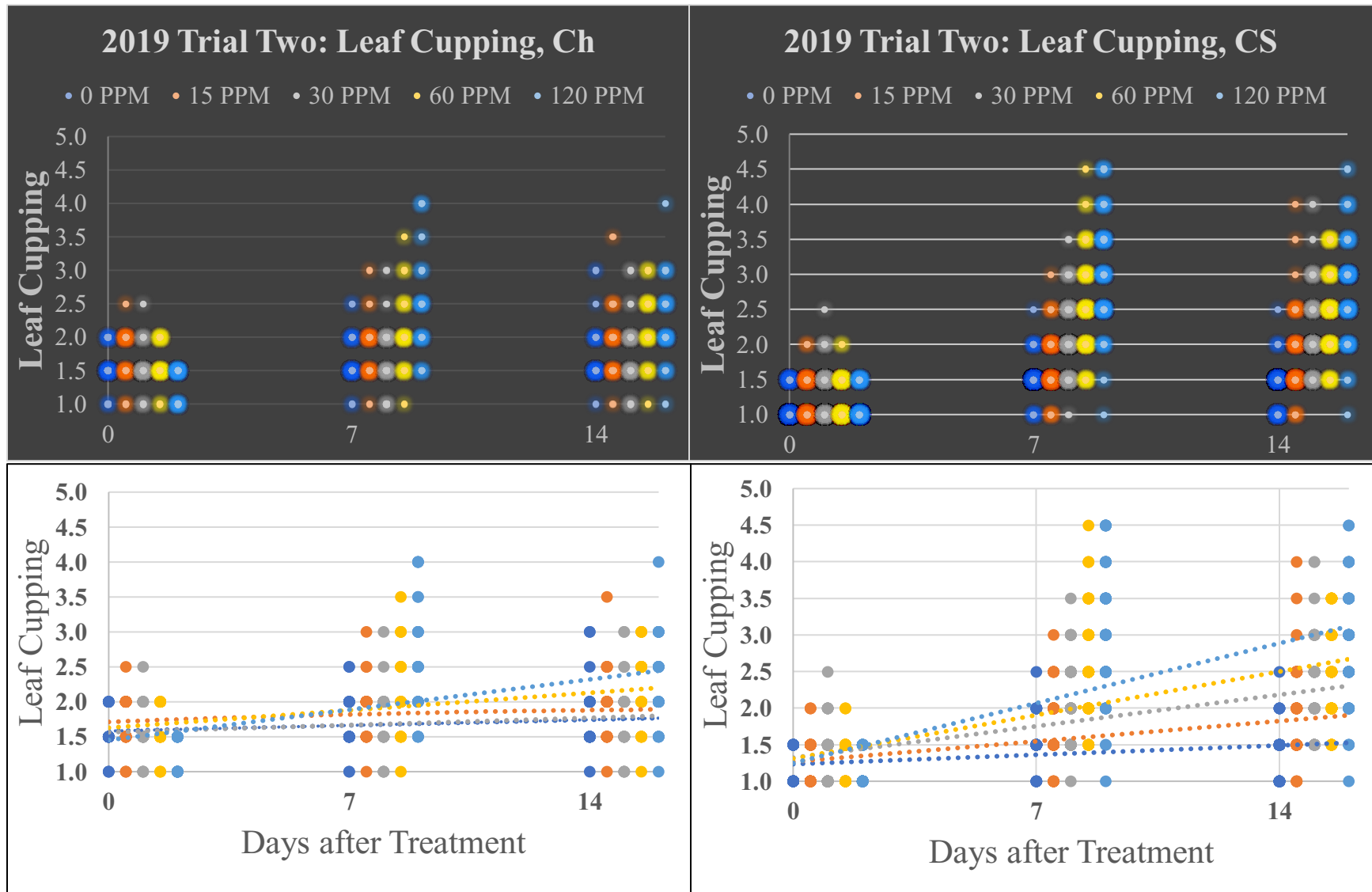


Figure 11. 2019 trial one leaf cupping score distribution and trends.

severe leaf cupping in both cultivars. For Ch, this increase is not pronounced with its 15 ppm treatment group, when examining its trendline, however each subsequent effect on the increase in dicamba rate can be visualized well with these linear regressions, with the slope of higher rate trendlines being greater than that of lower concentrations. When looking at CS trendlines, there is an even more compelling representation of the positive correlation between exposure to dicamba and the severity of leaf cupping injury. CS is more acutely sensitive to lower rates of dicamba than Ch, with each treatment level of CS showing greater disparities in leaf cupping over the control vines than Ch vines at both 7 and 14 DAT. The upper charts' relative frequency also demonstrates that a larger proportion of CS plants have higher leaf cupping scores than Ch vines in the weeks following dicamba exposure.

Visually, differences in the severity of leaf cupping injury to dicamba between cultivars were detectable at one week after the dicamba treatment. Photographs of dicamba exposed vines from each treatment group of both CS and Ch show the phenotypic responses of each cultivar under increasing concentrations of dicamba. Below, images taken 14 DAT from each cultivar are shown below in figure 12. Leaf cupping is apparent in all treatment levels. CS vines at even the lowest concentration are suffering from an increased distortion phenotype compared to Ch. As the concentration of dicamba increases, so does the corresponding damage. At 14 DAT, CS demonstrates more sensitivity to dicamba than Ch, as made apparent by the presence of severe leaf cupping in CS apparent as low as 30 ppm. The phenotype of the 120 ppm CS vine shows the shoot tip to be entirely wilted. In contrast, the 120 ppm Ch vine shows similar phenotypic leaf cupping injury to the 30 ppm CS vine. Aside from the leaf cupping phenotype, the effect of dicamba on shoot length of exposed grapevines was also recorded during this experiment. Figure

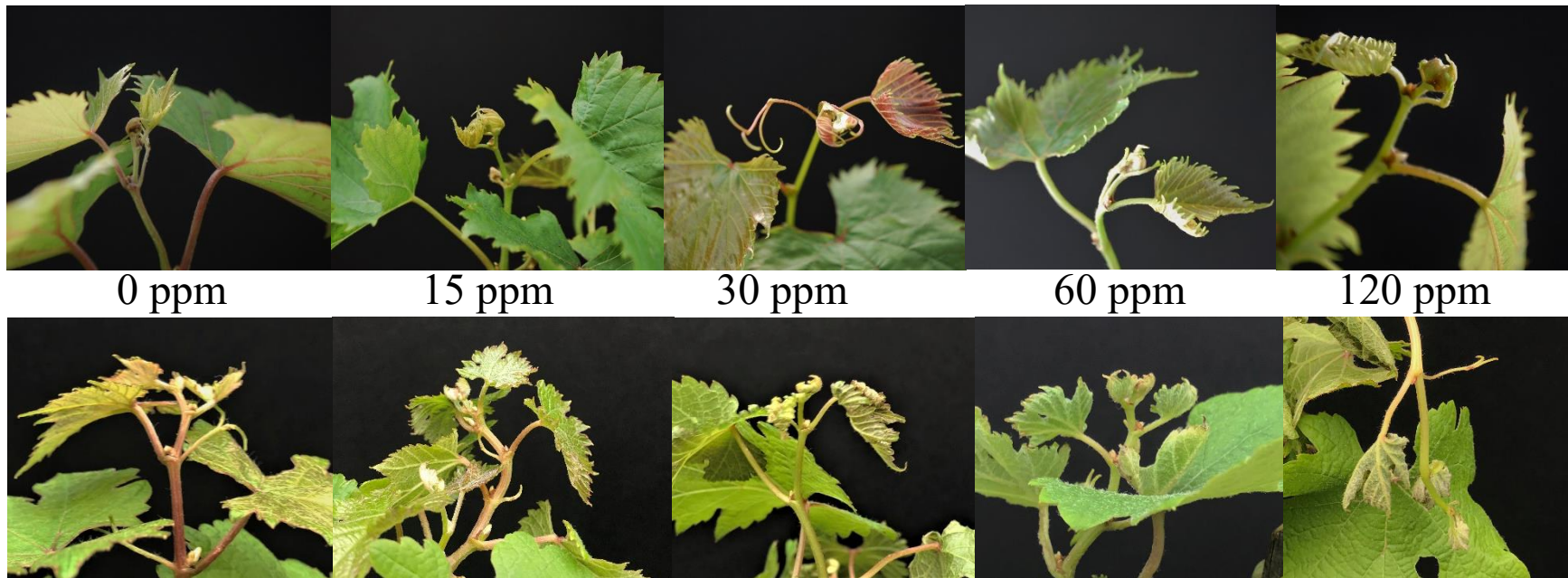


Figure 12. Photographs of chambourcin (top) and cabernet sauvignon (bottom) grapevines depict leaf cupping at different dicamba concentrations.

13 contains two bar charts which demonstrate the average shoot length of each cultivar at different days post treatment and for each treatment level sampled. Reductions in shoot length were noted in treated Ch vines. Compared to the increase in shoot length seen in the control group of about 5 cm in 14 days, all the treated Ch vines had their growth stunted with dicamba treatment. By 14 DAT, Ch control shoots grew significantly more than Ch treated vines. Conversely, for CS vines exposed to dicamba: few, if any differences in shoot length changes were noted between any levels of dicamba treatment. At 7 DAT, shoot length for the 120 ppm treatment group of CS actually grew more than the control, with no differences seen at 14 DAT. All CS vines increased a significant length between 0

and 14 DAT, with no differences seen due to treatment level. During the same period for Ch, no significant differences were found for changes in any group's mean shoot length.

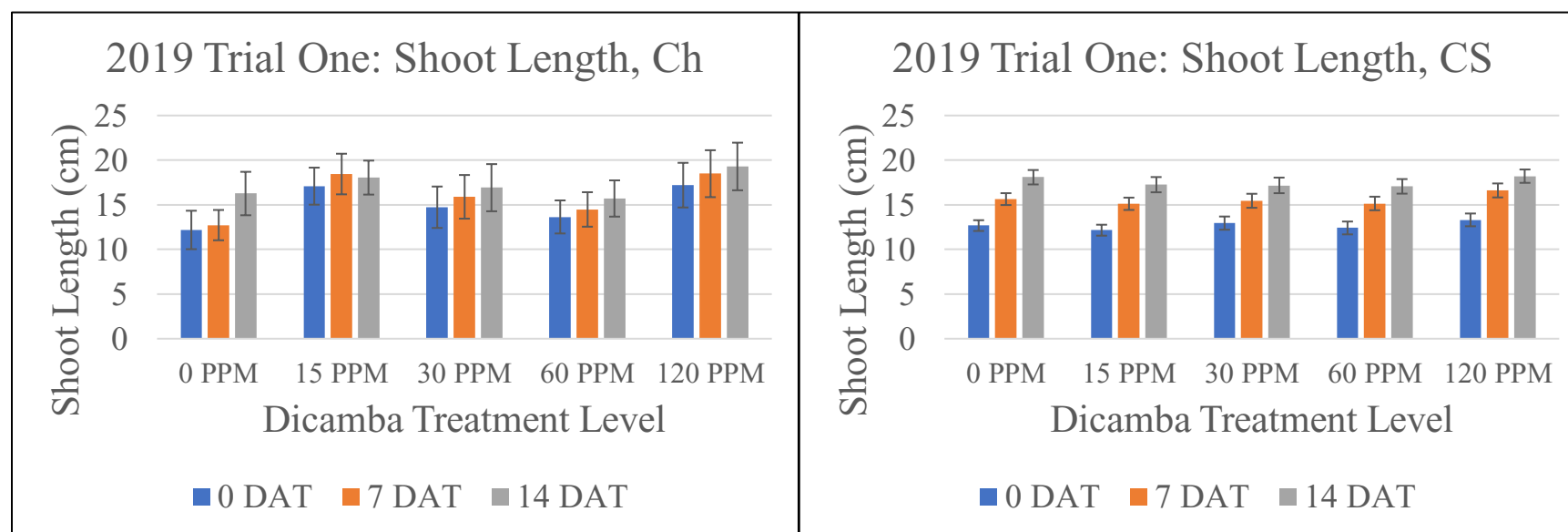


Figure 13. Group means of raw shoot length of treated vines over the course of 2019 experiment one.

2019: Experiment Two. Distribution and trends involving the leaf cupping phenotypes of Ch vines exposed to dicamba are summarized in figure 14. In this figure, dicamba as low as 15 ppm demonstrated elevated leaf cupping phenotypes compared to its control. Leaf cupping injury was more severe for the 30 and 60 ppm treatment groups. Differences between the effect of 30 ppm versus 60 ppm are negligible in Ch: in fact, 30 ppm exposed vines have a higher maximum leaf cupping score than the 60 ppm at all recorded dates. Leaf cupping injury increased weekly for all Ch treatment groups (and to a lesser degree, the control group), and did

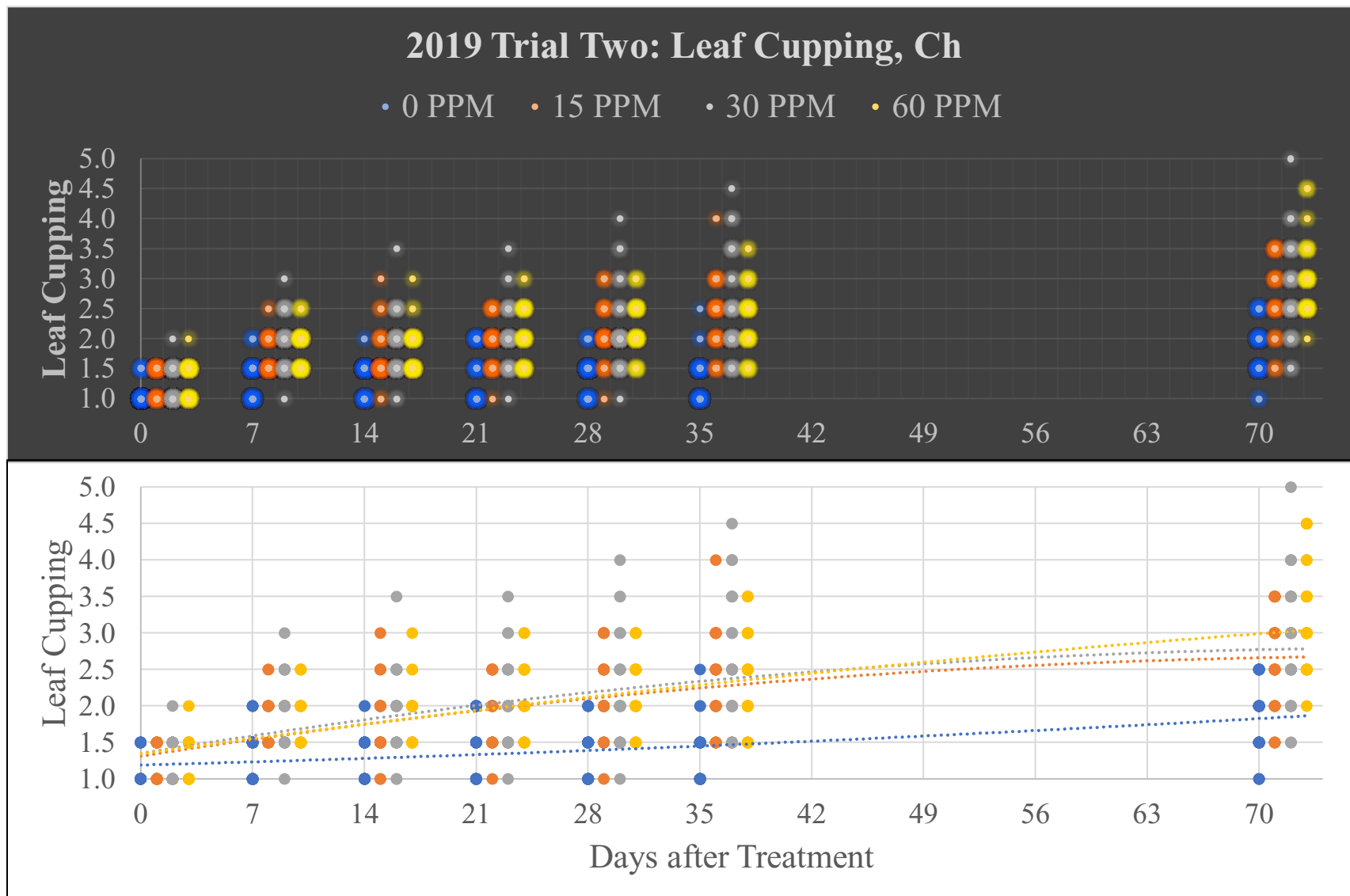


Figure 14. 2019 trial two leaf cupping score distribution and trends for chambourcin.

not stop for the entirety of the experiment. Of the dates scored, nearly each treatment group achieved maximal scores at 70 DAT, also where the largest phenotypic discrepancies between the control and the 60 ppm treatment group exist. Polynomial trendlines of these data points given show that dicamba, at all rate, affects the leaf cupping of Ch vine. While there are few differences between the leaf cupping injury trends of 15, 30, and 60 ppm dicamba-exposed vines, all three are markedly higher than the injury occurring in the control group. Increases in the leaf cupping rating of Ch control vines are small and occurs at a much slower rate than for dicamba treated vines.

A summary of similar data representing CS vines are shown in figure 15. Many differences exist between in the development of leaf cupping phenotypes between cultivars in this experiment. At every treatment level, 0 to 7 DAT saw a spike in leaf cupping for each group over the control. The 15 ppm showed substantial increase above the control at 7 DAT, yet was overshadowed compared to the 30 and 60 ppm treatments of CS. Both the 30 and 60 ppm treatment groups showed maximum leaf cupping injury in the first week post exposure to dicamba. Unlike the Ch vines, some CS treated plants actually decreased in leaf cupping severity over the course of the experiment. While the 15 ppm treatment level sees increases in leaf cupping severity up to 28 or 35 DAT, by 70 DAT, this number decreases. So, instead of continuing to develop phenotypic injury up to or even beyond 70 DAT like for Ch, this figure demonstrates that CS develops maximal injury soon after exposure, followed by a period where leaf cupping decreases in severity and the CS plants later show resilience post dicamba exposure. In fact, the greatest differences between the leaf cupping scores of the control and the 60 ppm treatment group for CS occurred at 7 DAT, versus 70 DAT for Ch. Trendlines in this figure representing CS vines show similar results as for Ch vines: the presence of dicamba, at any rate

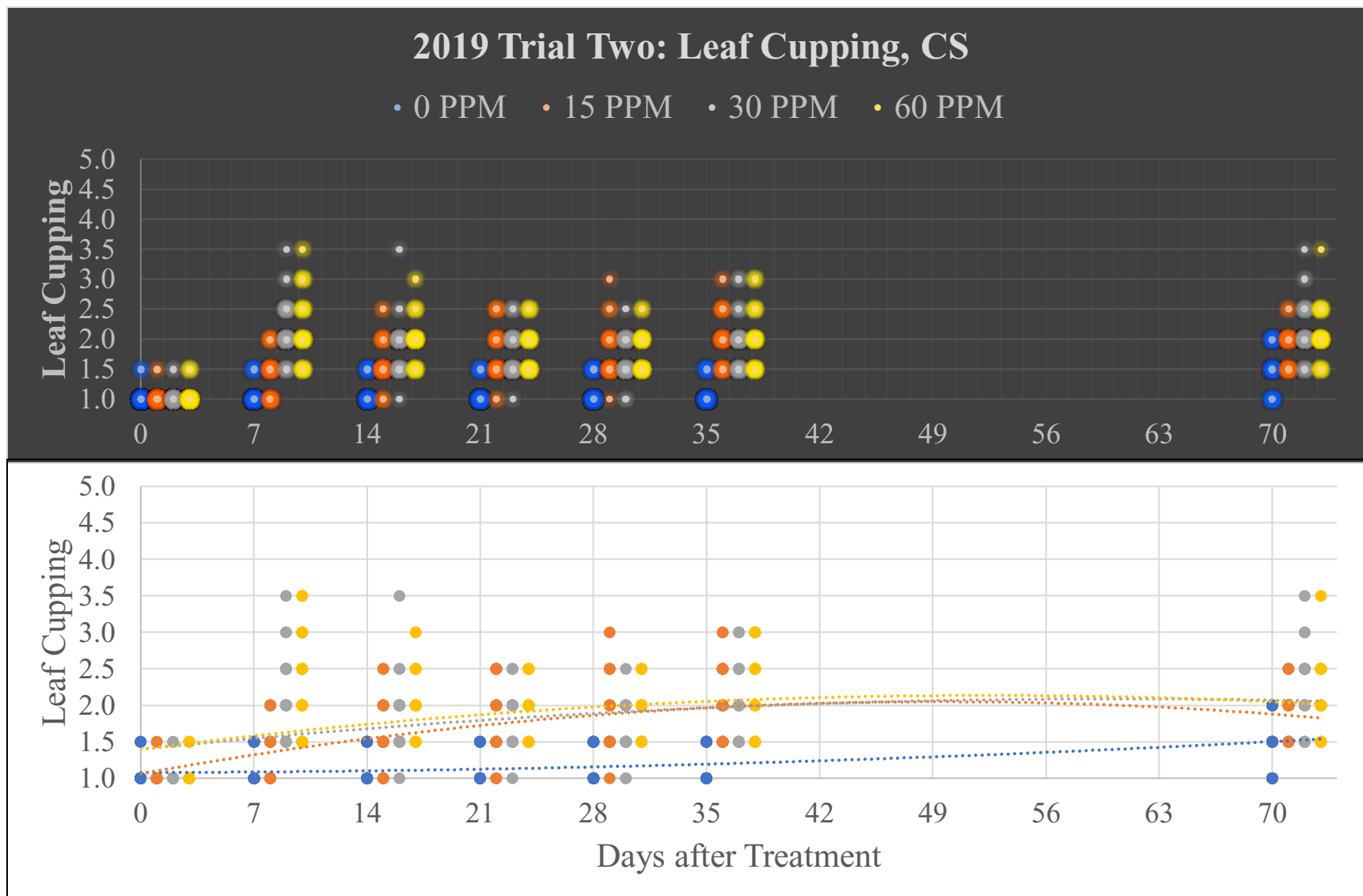


Figure 15. 2019 trial two leaf cupping score distribution and trends for cabernet sauvignon.

(tested here), is more injurious to the leaf cupping phenotype of the vine than the actual concentration, with little difference in the slope and intercepts of the trendlines representing the 15, 30, and 60 ppm dicamba exposed CS vines. While it remains important to note that actual scores of Ch and CS cannot be compared side by side, and natural differences in leaf cupping phenotypes exist independent of dicamba exposure, it is more important to note the extremes highs in leaf cupping achieved by Ch compared to CS. Several individual Ch plants reached leaf cupping scores of “4” and above at 35 and 70 DAT, where the maximum leaf cupping scores given to CS vines are “3.5”s. At 70 DAT, differences in the median phenotypic ratings of 60 ppm treatment groups and the control were about 2 full points (out of the possible 5) for Ch, and about 1 for CS.

The differential progression of leaf cupping between cultivars due to dicamba damage in this experiment are documented in the next several figures, including figure 16 below.



Figure 16. 2019: Spray drift simulation experiment 2, 21 DAT. A: Ch, 0 ppm; B: Ch, 15 ppm; C: Ch, 30 ppm; D: Ch, 60 ppm; E: CS, 0 ppm F: CS, 15 ppm; G: CS, 30 ppm, and H: CS, 60 ppm.

Combined, images of each cultivars' treatment groups over time will help depict the reality of the scores that the previous figures report. Figure 16 portrays representative vines of each cultivar and treatment level at 21 DAT. In these photographs, leaf cupping is most apparent at the meristematic locations: primarily at shoot tips and in the newest leaves nearest the shoot tips. Here, both 0 ppm treatments show little or no leaf cupping. At 15 ppm, leaf cupping is barely noticeable at the tops of each shoot, however is present. Differences between Ch and CS at 15 ppm are indiscernible at 21 DAT. At 30 ppm, more severe phenotypic responses to dicamba are shown as greater severity leaf cupping. At 60 ppm, leaf cupping is even more severe than at lower exposure concentrations, however differences between Ch and CS are still miniscule. Two weeks after the images taken for figure 16, figures 14 and 15 show that leaf cupping increases quite a bit for all Ch vines, whereas CS vines may increase slightly, but are generally the same as in previous weeks. Figure 17 shows representative vines of each cultivar and treatment level at

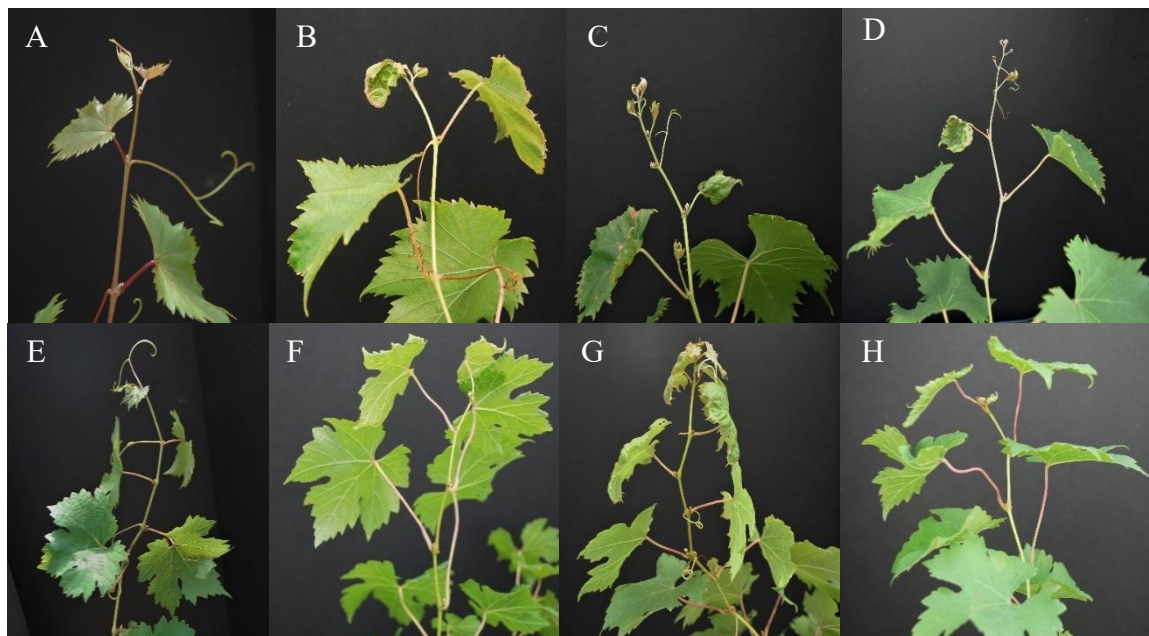


Figure 17. 2019: Spray drift simulation experiment 2, 35 DAT. A: Ch, 0 ppm; B: Ch, 15 ppm; C: Ch, 30 ppm; D: Ch, 60 ppm; E: CS, 0 ppm F: CS, 15 ppm; G: CS, 30 ppm, and H: CS, 60 ppm.

35 DAT. Control groups again demonstrate very little leaf cupping and generally have very healthy, flat leaves. As the concentrations increase, moving to the right, it becomes apparent that the vines at the top, Ch, are experiencing far more severe phenotypic effects than the bottom vines of CS. On CS, damage to dicamba exposed vines is apparent, especially at 30 ppm, however is not nearly as obvious as the damage done to Ch. At 15 ppm, the Ch vine shown already has at least one severely cupped leaf. The Ch vines here which were exposed to 30 and 60 ppm of dicamba have new leaves which have never fully developed due to severe, almost complete cupping. These leaves are closer to resembling curled up buds that are about to break than what a leaf should typically look like. Figure 18 shows images of vine phenotypes at 66

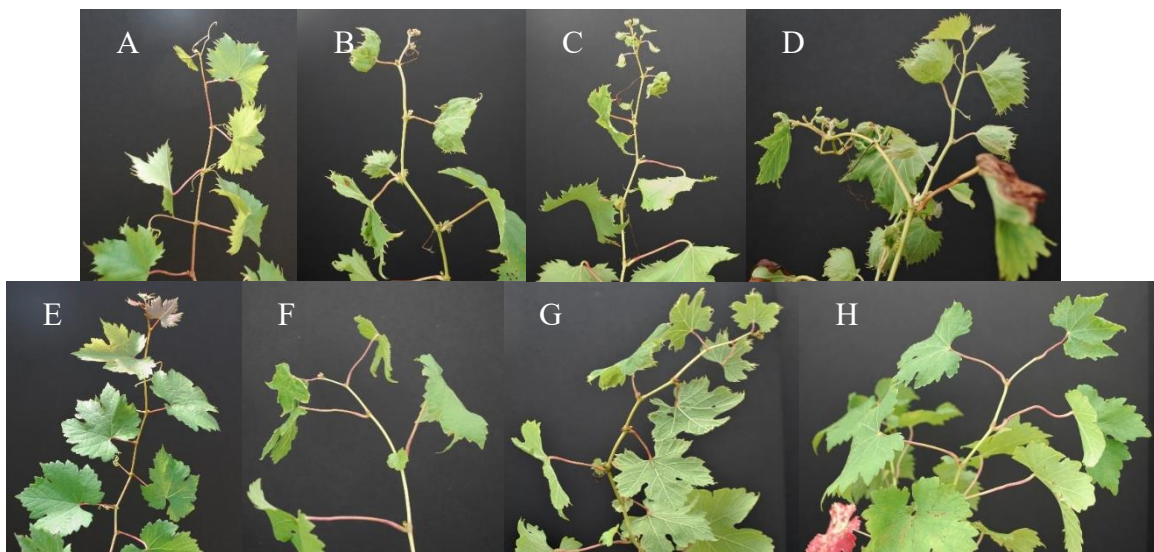


Figure 18. 2019: Spray drift simulation experiment 2, 66 DAT. A: Ch, 0 ppm; B: Ch, 15 ppm; C: Ch, 30 ppm; D: Ch, 60 ppm; E: CS, 0 ppm F: CS, 15 ppm; G: CS, 30 ppm, and H: CS, 60 ppm.

DAT. Like in the previous figures, the control groups at the left are completely undamaged by dicamba and have very low leaf cupping scores due to their flat leaves. Unlike the previous figures, CS vines also look completely undamaged by dicamba: at least compared to Ch vines. The images for CS vines exposed to dicamba treatments of 0, 15, 30, and 60 ppm are nearly

identical, with only hints of damage visible. CS vines appear less damaged at 66 DAT than at 35 DAT. On the contrary, Ch vines treated with dicamba appear the same if not worse at 66 DAT than 35 DAT. All three treatment groups display similar levels of leaf cupping damage, and all have leaves which appear to be almost completely cupped. More pictures of severely cupped leaves from this experiment are shown in the appendix. Shoot lengths of dicamba treated vines were also used to phenotype sensitivity to dicamba.

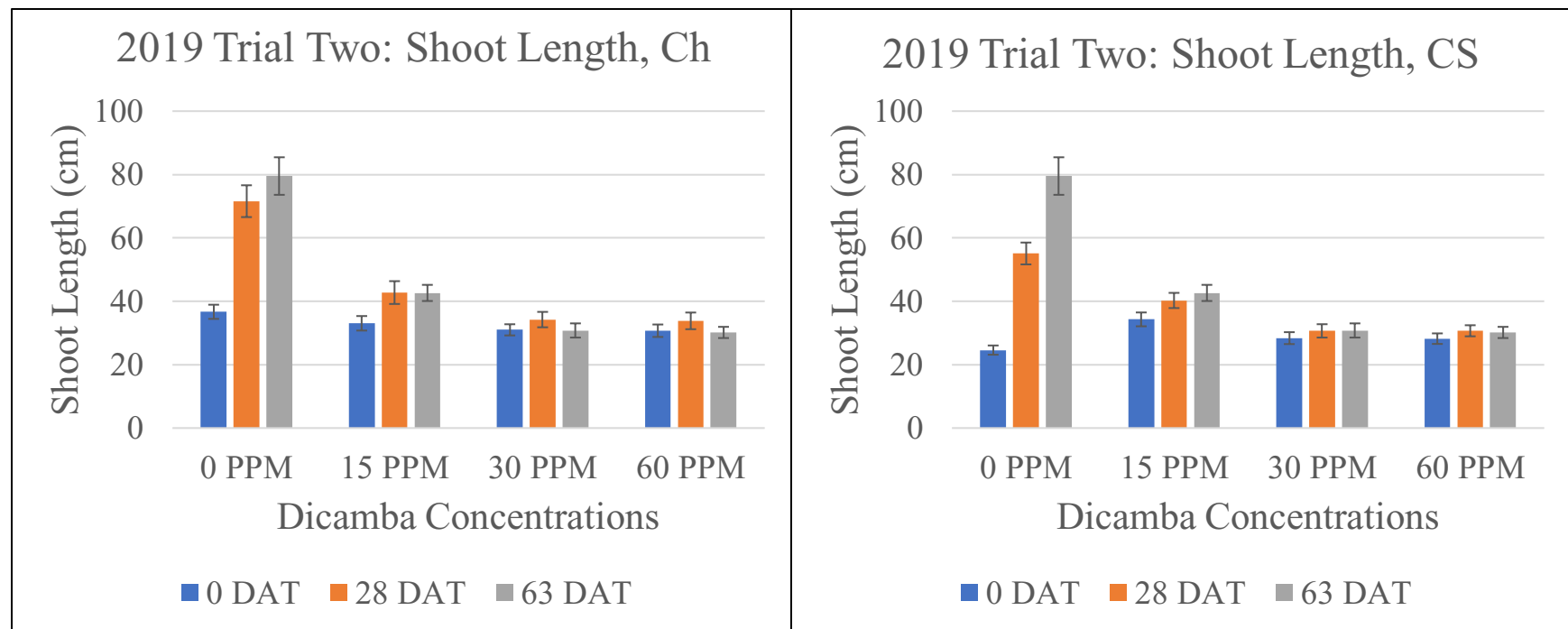


Figure 19. Group means of raw shoot length of treated vines over the course of 2019's trial two.

The group means of different cultivars and treatment levels over time are presented in figure 19. Across cultivars, the effect of dicamba on shoot length is consistent. All treatment groups, including the 15 ppm exposed vines, showed significantly lower group means for shoot length than their controls. Higher concentrations of dicamba exposure resulted in a more severe stunting of shoot length. No significant differences between cultivars were found when investigating vines subjected to similar dicamba treatments.

Detached Leaf Assays

Detached Leaf Assay One. Leaf cupping scores of Ch and CS are given in figure 20. According to this figure, all experimental groups experienced an increase in leaf cupping following excision from the mother plant and subsequent experimentation. At 1 DAT, after detached leaves were exposed to mild heat for 24 hours, detached leaves already demonstrated severe leaf cupping. Most control leaves have little to no cupping; however, it is possible for control leaves to achieve extremely high leaf cupping scores. Control leaves display a non-normal distribution, with high frequencies of leaves with little or no leaf cupping, as well as many leaves which are almost fully cupped/curled. Other groups with dicamba vapor treated leaves tend to have higher leaf cupping scores and are more normally distributed than their control counterparts. A positive correlation exists between the level of dicamba exposure to leaves and the development of leaf cupping of detached grapevine leaves. Over the 4-day experiment, leaves tested with higher dicamba concentrations showed elevated leaf cupping scores compared to lower dicamba, or no dicamba, treatments.

During this experiment, detached leaves were also given scores based on severity of

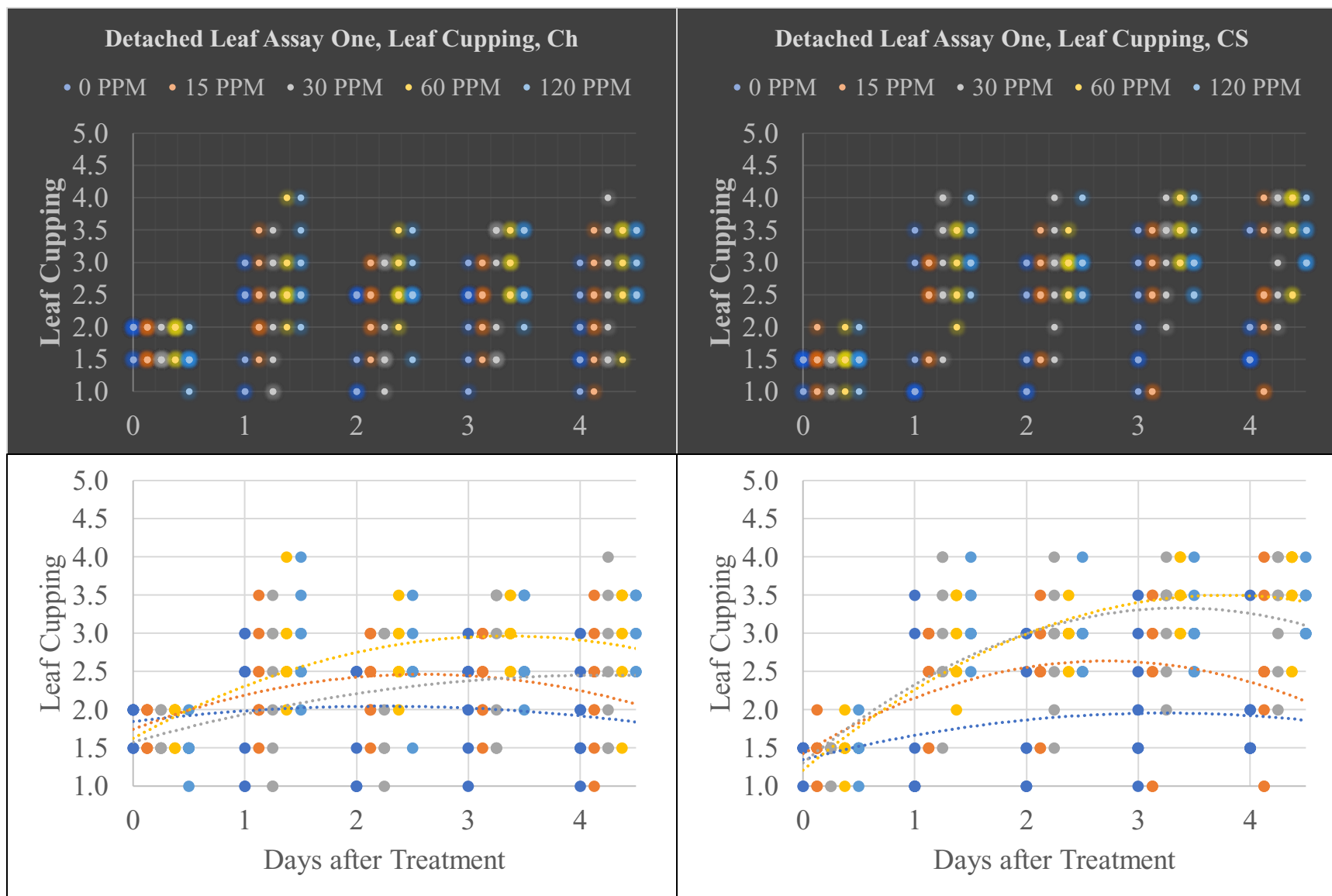


Figure 20. Detached leaf assay one: distribution and frequency of leaf cupping scores and their trends over time.

necrosis at 4 DAT, presented in figure 21. While leaves from all treatment groups display necrosis scores that rival those of higher concentrations, a greater frequency of high-exposure leaves is affected with necrosis in this experiment. The positive trendline seen in each cultivars' charts demonstrates the positive correlation between dicamba exposure and necrosis. While this correlation does exist, it is not necessarily strong: many leaves exposed to high levels of dicamba exhibit no necrosis, and vice versa, where leaves from the control group display high levels of necrosis sans exposure to dicamba.

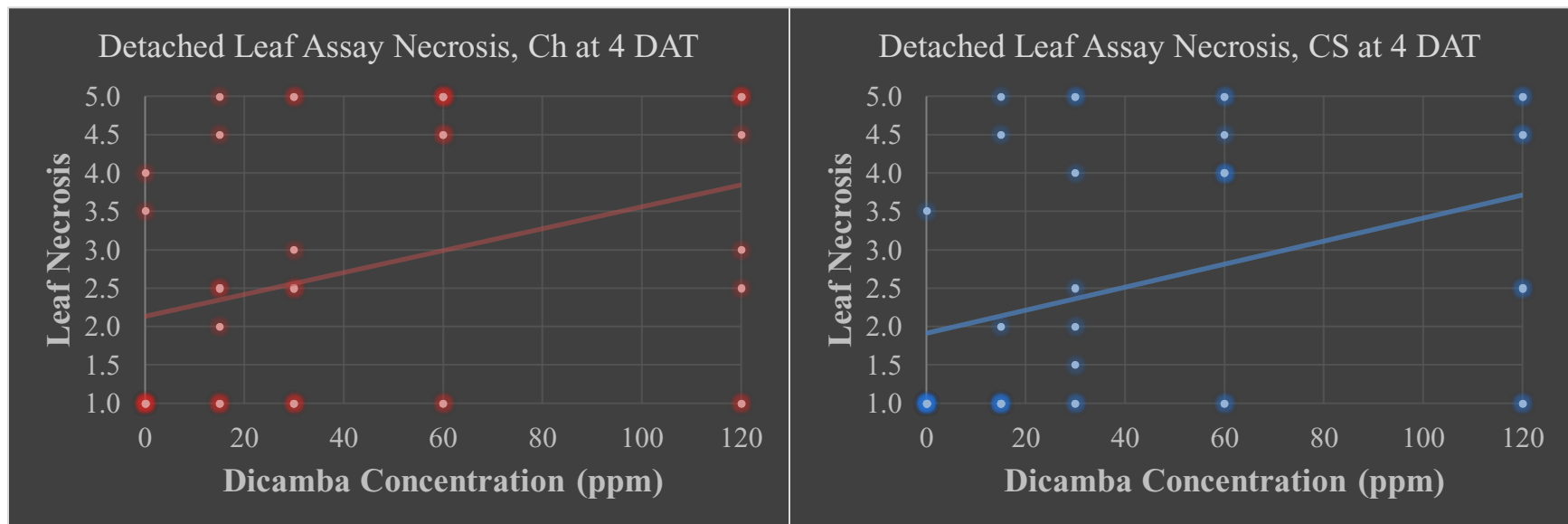


Figure 21. Detached leaf assay one: distribution of necrosis ratings under different dicamba concentrations.

Detached Leaf Assay Two. Very few physical effects of dicamba on detached leaves are documented in this experiment. The phenotypic scores of the different groups and cultivars at different DATs are presented in scatterplots in figure 22. After the initial 24-hour “incubation” period at room temperature, very few phenotypic changes occurred from pre-exposure recordings, and scores were largely the same at 0 and 1 DAT. This trend remained the same: few increases in leaf cupping are documented in the following days. In each cultivar, there are a handful of outliers that exist with dicamba-induced leaf cupping, but overall, few leaves experienced true, dicamba-induced leaf cupping phenotypes over the course of this experiment. Even at 4 DAT, the last day data were recorded for this assay, the leaf cupping scores of the control leaves are no different from any of the treated groups. The control group seem to have elevated leaf cupping scores at 4 DAT when compared to the dicamba treated leaves for both CS and Ch cultivars. Trendline analysis of this data indicate erratic patterns indicating the small fluctuations in the leaf cupping scores of the groups over the course of the experiment. While these day-to-day fluctuations exist, overall trends do not correlate incidence of leave cupping with increased dicamba exposure in this trial.

Detached Leaf Assay Three. This experiment exposed Ch and CS leaves treated with variable levels of dicamba vapor to a “heated” incubation period with leaves oriented abaxial side up. Under the heat with stomata facing upward, the environment proved too extreme for the leaves: all samples, regardless of cultivar or treatment level, were uniformly and completely shriveled and wilted prior to scoring at 1 DAT. No scores were recorded due to the uniformity of the damage across treatment groups and cultivar, nor were photographs taken to document the development of leaf cupping over time as in the other detached leaf experiments.

Detached Leaf Assay Four. Leaf cupping scores from this experiment are presented in

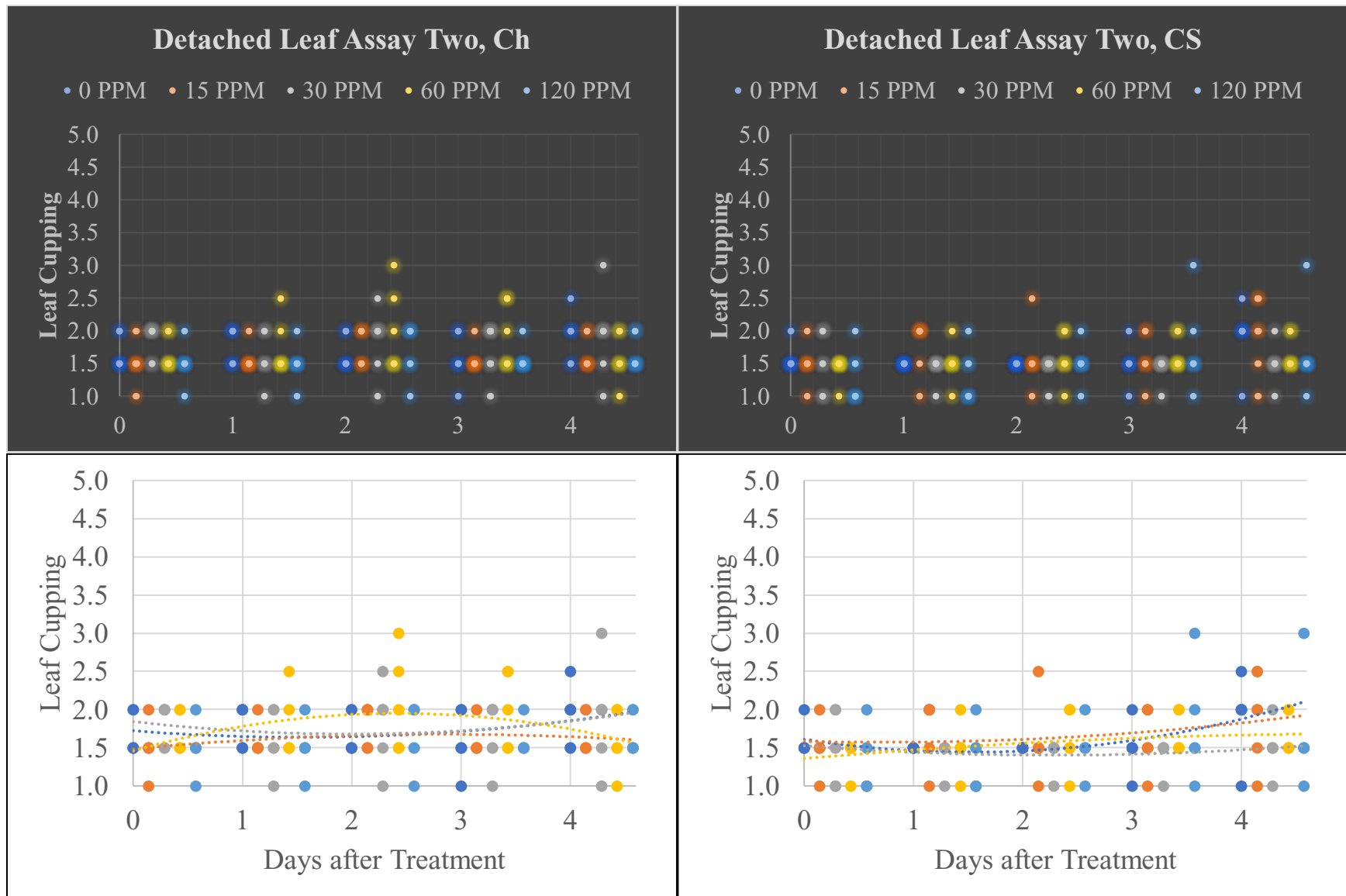


Figure 22. Detached leaf assay two: leaf cupping distribution and frequency in treatment groups over time.

figure 23. Leaf cupping in Ch leaves occurs at all levels, from the control up to 240 ppm exposed leaves. Overall, few differences in the treatment levels of Ch exist: high concentrations of dicamba elicit similar leaf cupping scores as the control. Leaf cupping increases well into 3 DAT for Ch, and may appear to continue to increase at and beyond 5 DAT. In contrast, CS control leaves do not respond with the same leaf cupping as Ch control leaves. Treated CS leaves show much greater discrepancies from the control than Ch leaves. Treated leaves of CS tend to have higher leaf cupping scores than the controls across the board. Leaf cupping of CS increases until around 2 DAT, whereafter leaf cupping scores decrease until the conclusion of the experiment at 5 DAT. The trendlines for these cultivars' data corroborate these findings; Ch trendlines are all jumbled together, with different treatment levels, including the control, intersecting and crossing one another's regression lines, indicating a similar effect from these treatments. CS trendlines in this experiment reveal that the control trended much lower in terms of leaf cupping compared to the other treatment groups, which all appear to have similar effects on leaf cupping in this trial.

Discussion

Spray Drift Simulations. The results of the spray drift simulation experiments suggest that both grapevine cultivars examined are sensitive to dicamba at low rates. In the initial 2018 trial, leaf cupping scores were similar among Ch and CS vines, as demonstrated in figure 6 and 8. Although, figure 6 also shows CS vines as slightly more sensitive to lower rates (20, 60 ppm) of dicamba than Ch vines. Distortion values in the 2018 experiments, represented in figures 7 and 9, show CS vines as being much more sensitive than Ch at all treatment levels, with the most apparent damage at the highest treatment level of 200 ppm. For all trials, the maximum

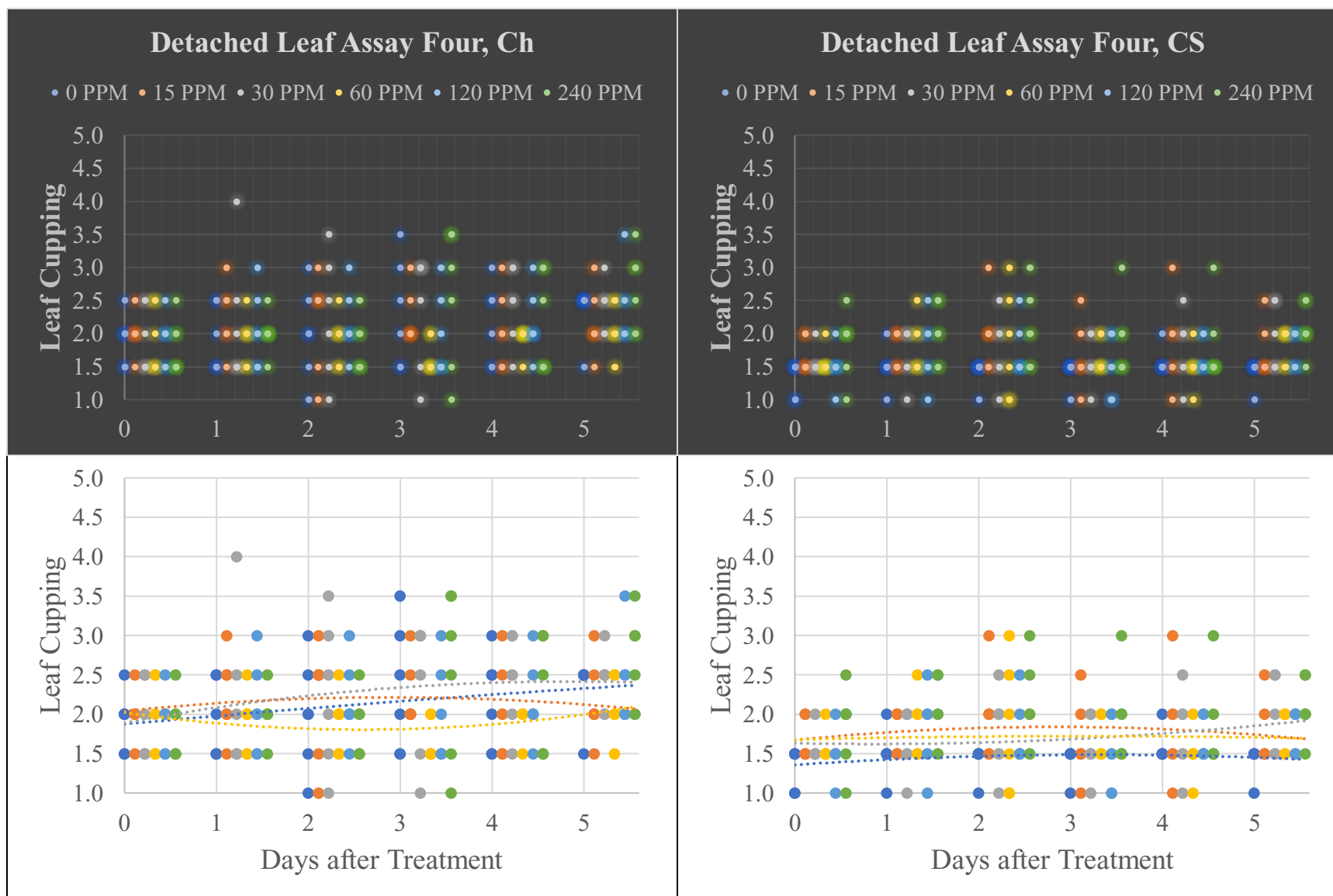


Figure 23. Detached leaf assay four: leaf cupping distribution and frequency in treatment groups over time.

distortion scores recorded were higher than the equivalent leaf cupping score. This is likely due to two factors: the first being that leaf cupping scores in the 2018 season are based on the healthiest leaf of the plant, while distortion is an average of the entire stem and each petiole. The second factor influencing the distortion and leaf cupping values is the time of the season. Performed in September and recorded through October 2018, the treated vines saw limited vegetative growth and were clearly in decline, coinciding with natural senescence. As leaf cupping is a physiological response of altered plant growth due to the auxinic effect of dicamba, less severe effects occur in periods of depressed plant growth. Due to this, more severe damage ratings were given from both cultivars for distortion and may be indicative of a phenotype better suited for late season exposure to dicamba than leaf cupping. However, the use of distortion of grapevine stems and petioles as a measure of dicamba damage may not be entirely valid. High distortion values were more common in CS vines, and even the Ch vines with the highest leaf cupping scores failed to also show high distortion values. It may be possible that stem and petiole distortion responses to dicamba are varietal dependent and may not be suitable for across the board as a measure of dicamba damage. For this reason, combined with the time intensive and subjective nature of scoring, distortion values were not recorded in subsequent trials performed the next season.

In the 2019 trials performed, CS vines initially demonstrated higher sensitivity to dicamba, manifest as leaf cupping damage, when compared to Ch as seen in figures 11, 14, and 15. Although in the second 2019 trial, the trend of CS leaf cupping scores outweighing Ch scores reversed several weeks after treatment, with Ch vines ultimately showing the greatest leaf cupping scores at 70 days after treatment. On the other hand, CS leaf cupping scores were at their highest at 7 days after dicamba exposure and decreased thereafter. Photos of Ch and CS vines in figures 16, 17, and 18 show the progression of each cultivars' phenotypes over time under different dicamba

regimes. CS vines generally appear to more rapidly respond to dicamba than Ch ones. Prior to 21 days after treatment, CS vines displayed greater leaf cupping damage than Ch vines, however in figure 16, the leaves look similar between cultivars at the same dicamba exposure levels. At 35 days after treatment, from figure 17, this trend becomes more visually apparent as Ch vines begin to appear more damaged than CS vines at the same treatment level. At 66 days post treatment, as displayed in figure 18, Ch vines appear to have even more severe leaf cupping at all treatment levels when compared to CS vines.

Shoot length measures initially point to CS vines being less impacted by dicamba than Ch. Ultimately however, differences between Ch and CS shoot lengths are insignificant. In trial one of 2019 (figure 13), shoot length measures show that dicamba, at all rates used, had no effect on the growth of CS vines, while it had a mild stunting of Ch vine growth. Later, figure 19 gives shoot length measures taken over the course of the second 2019 trial and shows that while higher rates of dicamba correlates with lower rates of shoot growth, there are no differences between cultivars in this study.

Overall, CS vines seem to have a recuperative ability or may simply react to and metabolize dicamba more rapidly than Ch vines. On the flipside, CS responds more acutely to dicamba damage than Ch. At the dicamba levels tested, CS appears to be more tolerant of dicamba damage than Ch, based on the conditions and appearances of the vines at the time of the termination of this experiment (70 days after treatment). Since CS vines reacted to low-dose dicamba exposure so quickly and severely compared to Ch, it remains unclear which variety would fare better under higher rates of exposure: whether it would immediately kill CS vines and give them less of a chance to recuperate, or if it would still ultimately show less damage than Ch. It is possible that Ch vines can buffer the binding of dicamba with auxin receptors through some unknown chemical

or process: effectively diluting, but also prolonging, the effects of dicamba on the vine. Alternatively, it is possible that dicamba is being metabolized unusually quickly in CS vines. As only two varieties have been tested in this experiment, it is impossible to determine which variety, if either, are closer to the “norm” for their species.

These findings go against the initial hypothesis that Ch vines, and European American hybrid vines in general, will outperform CS vines, and pure *Vitis vinifera* in general. While this held true for some trials and instances, it did not hold true for much of 2019 trial 2, which was the longest running experiment, as well as the one closest aligning with the time of the season at which dicamba would be used in the field. CS vines outperformed Ch vines for the majority of the experiment according to leaf cupping responses of the vines. These findings counter previous studies which have found European American hybrid grapes as generally having greater tolerance to synthetic auxins than pure *Vitis vinifera* grapes. As stated above, without further testing, it will remain unclear exactly which of these two cultivars, CS or Ch, are uncharacteristic of their species. Only when other varieties of these classifications can be tested alongside these two cultivars will it reveal whether CS contains a tolerance that most *Vitis vinifera* lack, or if Ch has greater sensitivity than most hybrids, or if this generalization fails to hold true under most conditions.

Future studies on this specific topic have much to learn from the trial and error of this project. The limited findings suggest that dicamba concentrations between 30 and 60 ppm, under the specific spray conditions used in the 2019 trial, are sufficient for causing visible dicamba damage suitable for rating the vines for tolerance characteristics, as previous literature has suggested. Visible damage to dicamba appears prior to seven days post exposure but may continue increasing in severity for several months thereafter, a much longer timeframe than many dicamba drift simulation studies. High levels of leaf cupping, distortion, and stunted shoot growth seem to

correlate well with dicamba exposure in grapevines, however are not direct indicators of plant health and performance. Further investigation into the effects of dicamba on plant biomass, photosynthetic capacity, fruit set, and berry composition are needed. Currently, nondestructive phenotyping of dicamba injury in plants over time is overwhelmingly subjective, with few quantitative measures outside of shoot length available to the average researcher. While many of the acute physical effects of dicamba on grapevines are documented, more research needs to be conducted on the true consequences of sublethal exposure to individual vines over time, and how different varieties fare under these conditions.

Many factors in this investigation converged to limit the success of this specific project, including restrictions on time, space, labor, equipment, previous knowledge, etc. With no restrictions and with the power of today's hindsight, this project would have seen far more grapevine varieties tested with larger sample sizes across a greater number of trials. Vines would also have been sprayed in open air where vapor drift is less likely to be contained for a prolonged period following exposure. Vines would be trained to a single shoot, or otherwise measured in a way which accounts for the entirety of the plant when phenotyping, as the systemic nature of dicamba allows the chemical to affect the entire plant, regardless of the exposure site. Following this logic, future research may achieve more reliable "doses" of dicamba in individual plants by applying small volumes of dilute dicamba directly on the leaves of the plant, as opposed to spray treatments that rely on the interception of spray droplets based on the surface areas of exposed plants. Unfortunately, any future researcher investigating this topic, or any other investigation of plants and plant development, will learn of the innumerable factors influencing ones' control over the natural world and plants themselves, even in the most controlled of settings.

Detached Leaf Assays. The detached leaf assays performed on Ch and CS reveal few differences between the cultivars' responses to dicamba. Trial one, with leaves incubated a greenhouse 24-hours post exposure, had the highest leaf cupping values of the three reported trials. Leaf cupping spiked across the board in the first day post exposure, with higher injury scores correlated to higher levels of dicamba exposure. No differences between Ch and CS were noted in this trial, and it was terminated after four days after treatment due to necrosis of the leaves interfering with the ability to score leaf cupping, combined with the appearance of unknown fungal structures on many leaves. Trial two's room temperature incubation with the detached leaves' petioles inside of cotton balls dipped in 2% sucrose prevented necrosis from overwhelming the leaves. This sugary, low-temperature treatment also prevented the development of leaf cupping. Whether these results are due from the lack of dicamba vapor forming, or rather a physiological preserving effect from the sucrose, is unknown. The cultivars responded similarly to one another following trial two's exposure to dicamba. Leaves were recorded with little or no leaf cupping with the experiment being terminated once the leaves began to bulge downwards under their own weight between the cotton ball and petri dish supporting them. Trial three fizzled out before its 24-hour greenhouse incubation post dicamba exposure was completed. Leaves, placed adaxial side down, fried in the afternoon sun and were uniformly wilted and browned during time of recording the first day after treatment's data despite a similar sucrose treatment as trial two. Only in the fourth trial do discrepancies between Ch and CS exist, where CS leaves are seen to have elevated leaf cupping scores as dicamba exposure increases while Ch treatment groups only display higher leaf cupping values than the control at five days after treatment when the control leaves started to go flaccid and limp. Leaf cupping occurs naturally after detachment from the mother plant as senescence begins, however this leaf cupping was especially pronounced for CS leaves treated

with dicamba. It is unclear why Ch control leaves would lose turgor at five days after, as is why dicamba may act to prevent this in treated Ch leaves. While CS has greater levels of leaf cupping than its control at every time point after exposure, Ch catches up to it at five days post treatment. Due to this, zero out of the three recorded treatments show Ch as having higher levels of tolerance to dicamba than CS for all treatment levels and days after treatment measured.

Only a few instances of severe leaf cupping phenotypes were reported during the entirety of the detached leaf experiment, even after several days post treatment. It may be reasonable to infer that the vapor levels of dicamba present within the vapor chambers is insufficient to manifest severe leaf cupping prior to the senescence of the leaf, where it becomes too necrosed or wilted to be accurately assessed on leaf cupping. More successful detached leaf assays of dicamba may be possible by increasing the amount of dicamba vapor reaching the plants, or by even allowing direct contact between dicamba and the detached leaves for rapid and ensured exposure to freshly detached leaves. It may yet be possible for detached grapevine leaves to respond to dicamba so drastically as live vines, with leaf cupping scores at 4's and 5's, however there is also the possibility that the altered proteins and conditions within the detached leaves are incapable of recreating such distinctive leaf cupping as in living plants. Using the proportion of necrotic tissue to rate the dicamba damage of a grapevine leaf may be possible as in trial one, however these necrotic results failed to occur in trials two and four. Instead of becoming necrosed, leaves in the latter two trials recorded were more likely to wilt and lose turgidity. Other phenotypic markers of senescence than wilting or necrosis, such as chlorophyll fluorescence and production of ethylene gas, may be more useful phenotypes in evaluating varieties for tolerance to dicamba, especially if the leaf cupping phenotype is inviable for such purposes.

Conclusions. The datasets above reveal that while CS is more acutely affected by exposure to simulated dicamba drift than Ch, it may also be more resilient to dicamba damage with maximum damage occurring after just two weeks and decreasing thereafter. Conversely, dicamba damage of Ch vines slowly increases over a period of months, to a point where its phenotypic severity overshadows anything seen on CS vines. The underlying physiological processes behind these phenomena are unknown, and it is unclear which cultivar has a lower lethal dose of dicamba on average. Both cultivars exhibit leaf cupping just days post treatment, with phenotypes best represented at concentrations of 30 and 60 ppm. In the end, more trials need to be performed to verify the replicability of these findings and more research needs to investigate how dicamba affects different varieties of grapevine and their berry characteristics over extended periods of time. Ultimately, a full understanding of how dicamba differently affect distinct varieties of grape will not be known until many more vines are treated and influenced by this chemical.

Next Steps: Breeding Tolerance with Molecular Markers. The final goal of this project is to breed and release a new cultivated variety of wine grape specifically bred, using molecular marker technology, for its dicamba resistance characteristics. This project will progress through the continued testing of Ch and CS vines, as well as other potential candidate varieties of interest. Eventually, if this further testing is to determine that one of these varieties contains a dicamba tolerance characteristic, the next step in the process is to determine the genetic basis of this trait: whether it is an inheritable trait or not. This requires the creation of a hybrid population for testing. If the trait is heritable, and segregates within the population (i.e. is not governed by a quantitative trait), then the trait is a good candidate for qualitative trait loci (QTL) analysis, followed by marker assisted selection (MAS). In order to perform QTL analysis, a linkage map of the hybrid “mapping” population must exist, where each hybrid individual must be screened using DNA

markers. With a sufficiently large hybrid mapping population, it is possible to locate DNA regions highly linked to the qualitative trait in question, known as QTLs. Once this proposed QTL region is identified with a high level of certainty to be (partially) responsible for the trait of interest, different seedlings can be tested for this trait based on the presence of DNA markers flanking this region. The presence of the closest possible DNA markers on both sides of the QTL of interest generally indicates the presence of this QTL, and the organism can be said with high levels of confidences to have the trait conferred by this QTL. The process of selecting seedlings to grow out and later use as potential breeding stock by the use of molecular markers is MAS. It is a process devised to identify desirable traits of plants and seedlings using only DNA, bypassing the need to reproduce the phenotype in question: saving time, labor, and space, by eliminating the need to grow out plants to maturity, or to perform intensive assays to elicit specific phenotypic conditions. This process is already underway at Missouri State's Fruit Experiment Station in Mountain Grove, MO. A hybrid mapping population of at least 306 individuals crossed between Ch and CS parents exists and is undergoing screening using hundreds and hundreds of simple-sequence repeat (SSR) DNA markers. After a linkage map is painstakingly produced from the SSR marker data, it would then be possible to screen the population for any known segregating phenotypic quality which has been assessed across the population. Other interspecific hybrid mapping populations between different parents also exist and are being screened for the same purpose as the ChxCS population. Contingent on finding a tolerance to dicamba in either Ch or CS (or any other parent used the other mapping populations), each individual of that mapping population will be screened for dicamba tolerance in order to determine its mode of inheritance. If it segregates nicely within the population as a qualitative trait, it will undergo QTL analysis, and any loci identified as responsible for this trait will be used for MAS in future breeding efforts. From here, breeding wine grapes for tolerance

combines science, art, and years of hard work with luck – the goal is to use MAS to screen plants for all QTLs for a desired cross, and home in on ones with the best characteristics and suitability for further breeding, and ultimately cultivar release.

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APPENDIX

Photographs of severely cupped grape leaves from 2019 drift simulation experiment 2. A, B, C, D, E, G, and I are all Ch vines. F, H, and J are CS vines. The selected vines represent the most severely cupped vines at 33 DAT.

