

The mechanism(s) and management of dicamba resistance in kochia (*Kochia scoparia*)

by

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B.S., China Agricultural University, 2008
Ph.D., China Agricultural University, 2013

AN ABSTRACT OF A DISSERTATION

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College of Agriculture

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Abstract

Kochia (*Kochia scoparia* (L.) Schrad), one of the most troublesome weeds in the North American Great Plains, including Kansas (KS), has become a significant concern in croplands ever since the evolution and spread of glyphosate resistance in this weed. Dicamba, an important synthetic auxin herbicide, is a useful substitute for managing glyphosate-resistant (GR) broadleaf weeds. As a result of extensive and intensive use, kochia populations have also developed resistance to dicamba. However, the precise mechanism(s) of dicamba resistance in kochia is still unknown. In the first part of this dissertation, the physiological, biochemical and genetic basis of dicamba resistance in dicamba-resistant (DR) kochia from KS was investigated. The results suggest that the mechanism of dicamba resistance in this kochia is not due to decreased absorption, reduced translocation or enhanced detoxification of dicamba. In contrary, reduced translocation of dicamba was found to contribute to the dicamba resistance in DR kochia from Colorado (CO). Further investigation of DR kochia from KS revealed a possible role of single nucleotide polymorphism (SNP) in *TIR1* (the receptor gene of auxin) in the dicamba resistance evolution. Genetic analyses of data from inheritance studies demonstrated that an incomplete dominant nuclear gene controls the dicamba resistance in kochia from KS. Also, it was found that the genes controlling dicamba resistance in kochia from KS and CO are not linked. Similarly, although, GR and DR traits were found to be controlled by two distinct single dominant genes, they appear to co-exist in many kochia populations from KS. Nonetheless, these two genes were also found not to be linked.

The second part of this dissertation focused on the development of reliable tools for the management of DR and/or GR kochia. The following experiments were conducted under greenhouse and field conditions in KS: a) the effect of temperature stress on the efficacy of

dicamba or glyphosate; b) efficacy of dicamba and glyphosate when applied in combination; and c) efficacy of dicamba when used as pre-emergence (PRE) herbicide. The results suggest that the efficacy of both dicamba and glyphosate on kochia can be improved when applied at cooler temperature conditions. Also, it was found that the dicamba and glyphosate tank-mix should not be recommended to manage kochia, especially DR kochia, due to significant antagonistic interaction when applied in combination. On the other hand, application of dicamba as PRE compared to the postemergence application, was found to improve kochia control including DR kochia. Overall, this dissertation provided several novel outcomes both in basic and applied aspects of dicamba resistance in kochia.

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Approved by:

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Dedication

This dissertation is dedicated to my dear wife, and our loving parents.

Chapter 1 - Literature Review

1.1 Weeds, Herbicides, and Herbicide Resistance in Weeds

A weed can be defined as “plant out of place” (WSSA, 1956) and weed infestation has been a major challenge in crop production (Hay, 1974). Among all other crop pests, weeds cause the most crop loss, followed by animals and pathogens worldwide (Oerke, 2006; Vats, 2015; Yaduraju, 2006). Weeds compete for light, moisture, and nutrient with crops (Vats, 2015) and if left uncontrolled can cause up to 52 and 49.5% of yield loss in corn (*Zea mays* L.) (Soltani et al., 2016) and soybean (*Glycine max* (L.) Merrill) (Soltani et al., 2017), respectively, resulting in 41.4 billion and \$16.3 billion annual economic losses in corn (Dille et al., 2015; Soltani et al., 2016) and soybean (Dille et al., 2016; Soltani et al., 2017), respectively.

The development of weed control methods almost co-exists with the history of agriculture (Bell, 2015; Hay, 1974). Earlier weed control methods including hand weeding, primitive tools to remove weeds, animal-powered implements, mechanically-powered implements, and biological and inorganic chemical methods have been reviewed extensively (Hay, 1974; Timmons, 1970). The discovery of the herbicidal properties of the phenoxyacetic acids in 1944 enabled the "Chemical Era of Agriculture" (Hay, 1974; Timmons, 1970; Vats, 2015). Since then, hundreds of organic compounds have been developed and commercialized for weed management (Appleby, 2005; Timmons, 1970; Vats, 2015).

Compared to other weed control methods, use of herbicides is time- and cost-effective, and more efficient with long-term weed control or suppression (Sharma and Gauttam, 2014). In the United States, over 90% of corn, soybean, and cotton (*Gossypium hirsutum* L.) have been treated with herbicides since the 1980s (Fernandez-Cornejo et al., 2014). A high percentage of

herbicide usage in other cash crops including rice, wheat, tomato, etc. has also been reported (Fernandez-Cornejo et al., 2014; Kniss, 2017). Herbicides accounted up to 65% of all pesticide expenditures, with an estimated cost of about \$5.1 billion in 2007 (Fernandez-Cornejo et al., 2014; Kniss, 2017). The most commonly used herbicides worldwide include glyphosate, atrazine, acetochlor, metolachlor, and 2,4-D (Fernandez-Cornejo et al., 2014).

Repeated and extensive application of herbicides exerts strong selection pressure on weed species and eventually leads to the evolution and spread of herbicide resistance in weeds (Heap, 2014; Vats, 2015). Herbicide resistance is, therefore, defined as “the inherited ability to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type” (WSSA, 1998).

The development of herbicide resistance in weeds as a result of extensive use of herbicides without proper stewardship is one of the major challenges for sustainable crop productivity (Délye et al., 2013; Vats, 2015). Introduction of herbicide-resistant crops in 1995, resulted in even more reliance on herbicides for weed control (Heap, 2014; Shaner, 2014). As a result, the evolution of resistance in the weeds increased rapidly. According to the international survey of herbicide resistant weeds, there are currently 486 unique cases of herbicide resistant weeds globally, with 253 weed species, including 147 dicots and 106 monocots in 92 crops in 70 countries. The global distribution of herbicide-resistant weeds is presented in Fig. 1.1. (Heap, 2017)

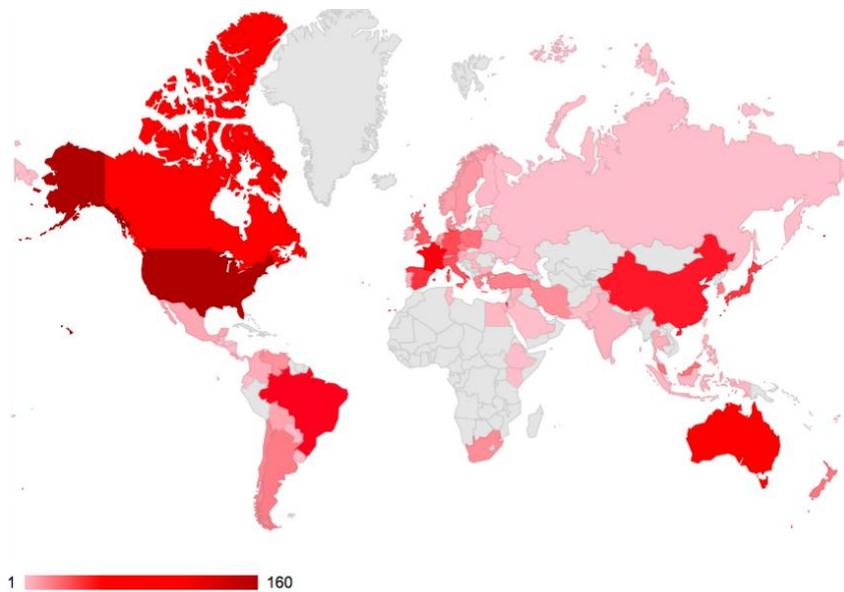


Figure 1.1 The number of unique cases of herbicide resistant weeds globally (Adopted from www.weedscience.org (Heap, 2017))

The types of herbicide resistance in weeds can be grouped into three categories. I) single herbicide resistance, meaning weeds are resistant to only a single mode of action of herbicide; II) cross-resistance, refers to weeds that are resistant to two or more herbicide classes within the same mode of action, or different modes of action of herbicides with a common mechanism and III) multiple herbicide resistance, defined as resistance to two or more herbicides with different modes of action with different mechanisms (Cobb and Reade, 2011). The evolution of multiple herbicide resistance is a challenge because such resistance limits the herbicide options for weed management in cropping systems. To date, 86 cases of multiple herbicide resistance in weeds including resistance to two or up to seven herbicide modes of action have been reported (Heap, 2017) (Fig. 1.2), e.g. rigid ryegrass (*Lolium rigidum* Gaud.), horseweed (*Conyza canadensis* L. Cronq.), Palmer amaranth (*Amaranthus palmeri* S. Wats), wild oat (*Avena fatua* L.), and kochia (*Kochia scoparia* (L.) Schrad.), etc. (Heap, 2017). The focus of this dissertation is to investigate the mechanism(s) and management of dicamba resistance in kochia, one of the most troublesome

weeds of Kansas, USA. Furthermore, multiple herbicide resistance in kochia is also common in the United States. Kochia, biology, management, distribution and evolution of herbicide resistance is discussed below.

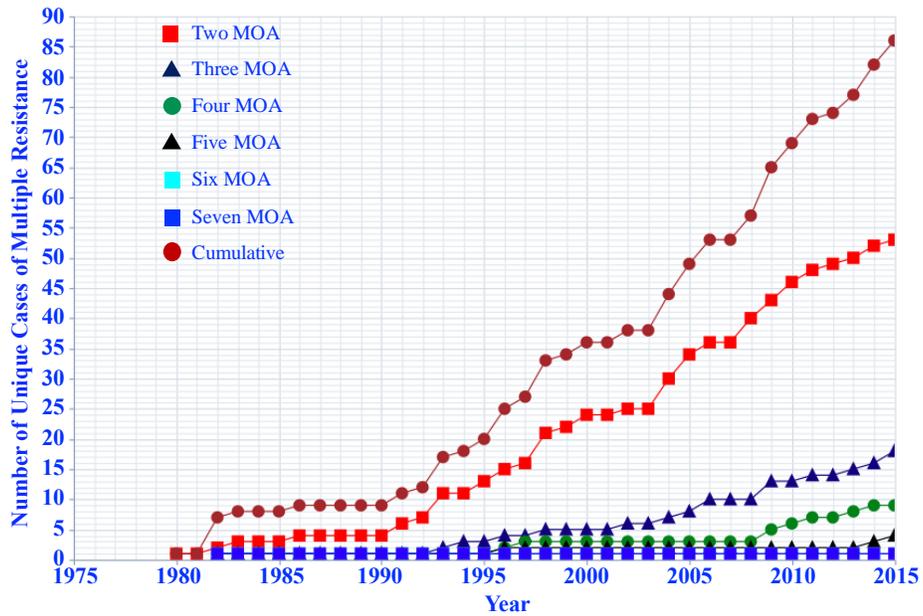


Figure 1.2 The reported occurrence of multiple resistance within the same weed population from 1975 to 2015. (Adopted and modified from www.weedscience.org (Heap, 2017))

1.2 Kochia Biology and Management

Kochia is an annual broadleaf weed species native to Eurasia and introduced as an ornamental to the Americas by immigrants in the mid to late 1800s (Friesen et al., 2009). This species soon naturalized and became an economically important weed of North America Great Plains in crop production systems in semiarid to arid regions, as well as pastures, waste areas, and roadsides (Dille et al., 2017; Friesen et al., 2009). Kochia can be used as a forage, especially in the early growing stage (Garduño, 1993). It is palatable to livestock, with nutrient value including protein content similar to that of alfalfa (*Medicago sativa* L.) (Finley and Sherrod, 1971) but can be toxic if it constitutes as the major percent of the diet or consumed at older growing stages (Sprowls, 1981). Kochia seeds can be a source of phytochemicals that are potentially beneficial to human health and has been used in Chinese medicine (Choi et al., 2002; Lee et al., 2013; Yoo et al., 2017). Kochia also can be used for phytoremediation of soils contaminated with heavy metals, hydrocarbons, or pesticides (Kafi et al., 2010; Moubasher et al., 2015; Perkovich et al., 1996). However, kochia is a troublesome weed in cropping systems in North America due to its tolerance to cold (Al-Ahmadi and Kafi, 2007; Anderson and Nielsen, 1996), heat (Khan et al., 2001), drought (Liu et al., 2008; Waldron et al., 2010), salinity (Friesen et al., 2009; Gul et al., 2010), and heavy metals (Zhao et al., 2015). In addition, with its ability to exert allelopathic properties (Hierro and Callaway, 2003; Karachi and Pieper, 1987; Lodhi, 1979) as well as its rapid growth under both cool and warm temperatures (Dille et al., 2012; Dille et al., 2017; Friesen et al., 2009) kochia can be highly competitive to crops. Also, this weed can disperse seeds by a tumbling mechanism facilitated by strong winds in the winter spreading seed across the Central Great Plains (Baker et al., 2008; Becker, 1978; Dille et al., 2017; Stallings et al., 1995a). This highly efficient mechanism of seed propagation enables kochia to reach the new

ecological niches and assists to become one of the fastest-spreading weeds in North America (Blackshaw et al., 2001; Forcella, 1985). The protogynous flowers (Guttieri et al., 1995; Stallings et al., 1995b) enables outcrossing, thereby, high genetic diversity in kochia (Mengistu and Messersmith, 2002), which contributes to rapid adaptation to new environments (Mengistu and Messersmith, 2002; Wiersma, 2012). Kochia has been listed as one of the top five problem weeds in the North American Great Plains cropping systems (Culpepper et al., 2017) including soybean, corn, sorghum (*Sorghum bicolor* (L.) Moench.), wheat (*Triticum aestivum* L.), and sunflower (*Helianthus annuus* L.) (Kumar and Jha, 2015; Osipitan, 2016; Wolf et al., 2000).

One of the most effective practices for kochia management includes tillage (Waite, 2010), because I) disturbance of the soil surface by tillage can bury most of the kochia seeds in deeper soil and reduce the seed germination, and also can prevent season-long kochia emergence (Zorner et al., 1984); and II) the size of the seed bank kochia in deeper soil will be reduced rapidly due to short seed longevity (Burnside et al., 1981; Thompson et al., 1994). However, wide adoption of no-till agriculture to prevent soil erosion and conserve the soil moisture (Pimentel et al., 1995), tillage is not a viable option for kochia control. Therefore, use of herbicides has been the major means of kochia management, including preplant (PP), preemergence (PRE) and postemergence (POST) application of herbicides.

Commonly used herbicide active ingredients for kochia management include glyphosate, dicamba, atrazine, mesotrione, and others (Thompson et al., 2018). However, prolonged and repeated herbicide application resulted in the evolution of resistance in kochia including multiple resistance to different modes of action herbicides (Heap, 2017; Osipitan, 2016; Varanasi et al., 2015).

1.3 Herbicide Resistance in Kochia

To date, 54 unique cases of herbicide-resistant kochia biotypes have been reported in Canada, Czech Republic, and the United States (Heap, 2017), including resistance to four herbicide modes of action: acetolactate synthase (ALS)-inhibitors (WSSA group 2), synthetic auxins (WSSA group 4), photosystem (PS) II-inhibitors (WSSA group 5), 5-enolpyruvate-shikimate-3-phosphate synthase (EPSPS)-inhibitor (WSSA group 9) (Heap, 2017; WSSA, 2017). In 1979, the first case of kochia resistance to PSII-inhibitors was reported from KS, USA (Johnston and Wood, 1976). In 1980s, due to rapid and wide adoption of ALS-inhibitors, 16 cases of kochia resistant to ALS-inhibitors were reported in less than 10 years (Green, 2007; Warwick et al., 2008) including an initial report in North Dakota, in 1987 (Saari et al., 1990; Shaner, 1997). Overall, in North America, ALS-resistant kochia has been reported in 23 states and provinces (Fig. 1.3). Later, in late 1990s, glyphosate (EPSPS inhibitor) was widely applied in cropping systems, primarily as a result of widespread adoption of glyphosate-resistant technology in corn, soybean, cotton, canola (*Brassica napus*), etc., which provides a weed control program that is simple and effective to a broad spectrum of weeds without injuring crops or restricting crop rotation (Carpenter and Gianessi, 2000). In 2005, the first glyphosate-resistant kochia population was found in KS, USA, which was documented and reported in 2007 (Heap, 2014; Wiersma et al., 2015). Soon after, 15 more cases of glyphosate-resistant kochia populations have been reported (Fig. 1.3) across the Great Plains of North America (Heap, 2017). Synthetic auxin herbicides are the first group of herbicides commercialized for use in agriculture and have been in use for more than 70 years to selectively control broadleaf weeds. Especially, dicamba, a synthetic auxin has been found to be an option to manage kochia after the widespread incidence of glyphosate resistance. Nonetheless, resistance to dicamba has also been reported in several

states and provinces in North American Great Plains (Heap, 2017) after the initial case in MT, USA in 1994 (Cranston et al., 2001; Heap, 2017).

Resistance to a single mode of action of herbicide is common in kochia. However, multiple herbicide-resistant kochia is also emerging rapidly. To date, 11 out of 54 herbicide-resistant kochia populations are resistant to two or more modes of action herbicides (Heap, 2017). Particularly, a single kochia population from Kansas has been found to be resistant to four modes of action (Heap, 2017; Varanasi et al., 2015).

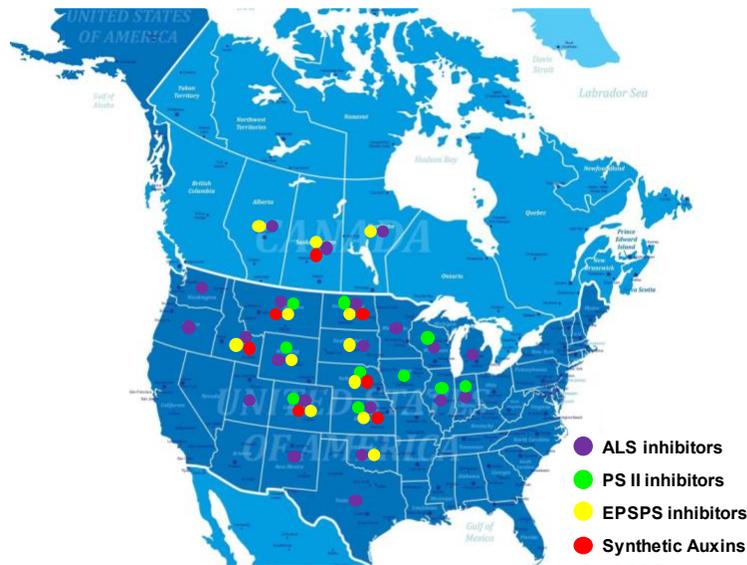


Figure 1.3 Distribution of herbicide-resistant kochia in North America. (Adapted from www.weedscience.org (Heap, 2017))

1.4 Mechanisms of Herbicide Resistance

In general, the processes that lead to plant death in response to herbicide application include: I) herbicide penetration into the plant via leaf or root absorption after foliar or soil application; II) herbicide movement to plant tissues/organs via apoplast and/or symplast pathways to reach the target-site; and III) finally, the interaction of herbicide molecules with target site resulting in irreversible abnormal biochemical and physiological reactions, which ultimately trigger the death of the plants (Ashton and Crafts, 1973; Audus, 1964; Cobb and Reade, 2011; Devine et al., 1992).

Weed species can evolve mechanism(s) to cope with or reduce the damage caused by herbicides. Any alteration to the processes described above can result in the evolution of resistance to herbicides in weeds (Gasquez, 1997; Jasieniuk et al., 1996). The mechanisms of weed resistance to herbicides can be grouped into two major categories (Délye et al., 2015; Holt et al., 1993; Yu and Powles, 2014). In the first type, weeds can develop a specific mechanism(s) to prevent the herbicide molecules from reaching the target-site, by altering the absorption, translocation, or detoxification of the herbicide molecules. This type of mechanism is referred as a non-target-site mechanism of herbicide resistance. The second mechanism includes, weeds that exhibit alterations in the target site, resulting in lack of herbicide binding or reduced interaction with the target.

1.4.1 Non-Target-Site Resistance Mechanisms

Three different mechanisms can be involved in non-target-site resistance to limit the amount of herbicide to reach the herbicide target, which includes reduced absorption, restricted translocation, and increased metabolism of the herbicide molecules (Powles and Yu, 2010; Yuan et al., 2007). Restricted translocation of herbicide as a mechanism of resistance to herbicide has

been reported in several populations of glyphosate-resistant horseweed (Feng et al., 2004; Ge et al., 2010), hairy fleabane (*Conyza bonariensis* L.) (Moretti et al., 2013), ryegrass (Wakelin et al., 2004; Yu et al., 2009; Yu et al., 2007; Yu et al., 2004), and johnsongrass (*Sorghum halepense* (L.) Pers.) (Vila-Aiub et al., 2012) and also in paraquat-resistant populations of barley (*Hordeum leporinum* (L.) Link) (Preston et al., 2005), hairy fleabane (Moretti, 2016), capeweed (*Arctotheca calendula* (L.) Levyns) (Soar et al., 2003), horseweed (Moretti, 2016; Váradi et al., 2000), and ryegrass (Powles and Holtum, 1994; Preston et al., 2009; Yu et al., 2007). Reduced translocation has also found to contribute to dicamba resistance in kochia (Pettinga et al., 2017) and 2,4-D resistance in wild radish (*Raphanus raphanistrum* L.) (Goggin et al., 2016).

The other major category of non-target-site resistance mechanism is herbicide detoxification (i.e., metabolism-based) that endowed as a result of four-phase chemical reactions: Phase I involving oxidation, typically facilitated by the catalytic activity of cytochrome P450 monooxygenases (P450s) or mixed function oxidases. Phase I detoxification exposes certain functional groups to further metabolism in phase II resulting in the conjugation of the oxidized/activated xenobiotic product usually with a thiol or sugar molecule. This can enable the recognition of the product to Phase III transporters, which includes sequestration of molecules into vacuole or extracellular spaces in the plant, which is most commonly carried out by adenosine triphosphate (ATP)-binding cassette (ABC) transporters. The phase IV detoxification process includes further degradation to less toxic compounds (Bartholomew et al., 2002; Martinoia et al., 1993; Sandermann, 2004; Yuan et al., 2007). The four major groups of enzymes known to be involved in non-target-site herbicide resistance include P450s, glutathione-S-transferases (GSTs), glycosyltransferases, and ABC transporters (Powles and Yu, 2010; Yuan et al., 2007).

Metabolism-based herbicide resistance to PSII-, ALS-, acetyl CoA carboxylase (ACCase)-inhibitors (WSSA group 1), and synthetic auxins, has been reported in several weed species, including velvetleaf (*Abutilon theophrasti* L.) (Anderson and Gronwald, 1991; Gronwald et al., 1989), smooth amaranth (*Amaranthus hybridus* (L.) Amach.) (Manley et al., 1999), Palmer amaranth (Nakka et al., 2017a; Nakka et al., 2017b), common waterhemp (*Amaranthus tuberculatus* (L.) Moq.) (Figueiredo et al., 2017), downy brome (*Bromus tectorum* L.) (Park et al., 2004), blackgrass (*Alopecurus myosuroides* (L.) Huds.) (Letouzé and Gasquez, 2003), rigid ryegrass (Cocker et al., 2001; Vila-Aiub et al., 2005), Italian ryegrass (*Lolium multiflorum* (L.) Lam.) (Gronwald et al., 1992), wild oat accession (*Avena sterilis* L.) (Shimabukuro et al., 1979), littleseed canarygrass (*Phalaris minor* (L.) Retz.) (Chhokar and Malik, 2002), late watergrass (*Echinochloa phyllopogon* (Stapf) Koss.) (Bakkali et al., 2007; Yasuor et al., 2009; Yun et al., 2005), chickweed (*Stellaria media* (L.) Vill.) (Coupland et al., 1990; Saari et al., 1992), large crabgrass (*Digitaria sanguinalis* (L.) Scop.) (Everman et al., 2009), and wild mustard (*Sinapis arvensis* (L.) Sinar.) (Peniuk et al., 1993; Veldhuis et al., 2000), etc. Metabolism-based herbicide resistance is particularly threatening, because the weed populations can potentially detoxify other classes of herbicides, including never-used herbicides or newly developed herbicides (Délye et al., 2011; Ghanizadeh et al., 2017; Powles and Holtum, 1994; Powles and Preston, 2006; Yuan et al., 2007).

1.4.2 Target-Site Resistance Mechanisms

Four possible mechanisms can be involved in target-site herbicide resistance: a) altered target-site, b) target gene over expression (increased synthesis of target protein), c) target gene amplification, and d) regulatory changes in the target-site (Délye et al., 2013; Nakka, 2016; Powles and Yu, 2010). The most common target-site mechanism of herbicide resistance is due to

mutation(s) in the herbicide target gene resulting in the modification of tertiary and/or quaternary target protein structure to prevent the ligand (herbicide)-receptor (target protein) binding interaction and keep the normal protein function (e.g. enzymatic activities) at the same time. For example, mutations in the herbicide target genes such as, *psbA*, *ALS*, *ACC*, and *EPSPS* can confer resistance to PS II-, ALS-, ACCase- or EPSPS-inhibitor herbicides, respectively (Déllye et al., 2013; Heap, 2017; Powles and Yu, 2010). Although less frequent, an entire codon deletion in the target gene is also known to endow resistance to protoporphyrinogen oxidase (PPO)-inhibitors (WSSA group 14) in common waterhemp (Lee et al., 2008; Patzoldt et al., 2006; Thinglum et al., 2011) and Palmer amaranth (Salas et al., 2016; Salas-Perez et al., 2017). Also, it has been reported that different amino acid substitutions at the same codon of *ALS* gene can confer different levels of resistance to different spectrums of ALS-inhibitors in wild radish (Han et al., 2012), whereas, accumulation of mutations at non-consecutive codons conferred higher level of glyphosate resistance in goosegrass (*Eleusine indica* (L.) Gaetner) (Jalaludin et al., 2013).

Target gene amplification is also a novel mechanism conferring herbicide resistance in weeds, especially for glyphosate. The first such case was reported in glyphosate-resistant Palmer amaranth, which had more than 100 copies of *EPSPS* gene distributed throughout the genome (Gaines et al., 2010). Later, such mechanism has been reported in a number of other glyphosate-resistant weed species, such as kochia, common waterhemp, spiny amaranth, and Italian ryegrass, etc. (Chahal et al., 2017; Jugulam et al., 2014; Kohrt et al., 2017; Mohseni-Moghadam et al., 2013; Nandula et al., 2014; Salas et al., 2012; Sosnoskie et al., 2011; Varanasi et al., 2015; Wiersma et al., 2015). Till recently, the herbicide target gene amplification has been shown to confer only glyphosate resistance, and one of the reasons for this occurrence may be because this

mechanism may be more “cost-effective” compared to other mechanisms in these weed species to withstand glyphosate selection (Bradshaw et al., 1997; Tranel, 2017). However, amplification of the *ACCase* gene conferring resistance to ACCase-inhibitors was recently reported in large crabgrass (Laforest et al., 2017). In this large crabgrass population 6- to 8-fold amplification of the *ACCase* gene and 4.4-10.3 times more expression of the ACCase transcript relative to a known sensitive population were detected without any known mutation in the gene. As mentioned above, enhanced metabolism-based resistance to ACCase-inhibitors is also common. Therefore, the assumption that the herbicide target gene amplification-based resistance evolution appears to be not specific to glyphosate (Laforest et al., 2017; Tranel, 2017), and mechanism of gene amplification may not be as rare as it was assumed earlier, especially there are only a small portion of the documented herbicide-resistant weeds have been tested for this mechanism of resistance to herbicides.

The focus of this dissertation was to investigate the mechanism(s) and management of dicamba resistance in kochia, and the following sections provide a more detailed description of the mode of action and mechanism of resistance of synthetic auxin herbicides, such as dicamba.

1.5 Mode of Action of Synthetic Auxin Herbicides

Synthetic auxin herbicides have been in use for more than 70 years around the world, primarily because of their high efficacy, selectivity, low toxicity, and low costs, (Peterson et al., 2016). When used at low concentrations, these herbicides mimic several physiological and biochemical responses as that of the natural plant hormone – indole-3-acetic acid (IAA), which is referred as the “master hormone” in higher plants (Grossmann, 2010; Ross et al., 2001). IAA is virtually involved in every aspect of plant growth and development, including cell division, cell elongation, vascular tissue development, tissue differentiation, organ formation, senescence, apical dominance, tropic responses. Auxins also interact with other phytohormones to form a complex network to regulate plant growth and development (Davies, 2013). The synthetic auxin herbicides have a similar chemical structure (Fig. 1.4) as IAA. When used at low concentrations, 2,4-D can stimulate embryo development *in vitro* (Dudits et al., 1991). Furthermore, low concentrations of synthetic auxin herbicides are also used in plant biology research to study the binding affinity alterations among the auxin target transport inhibitor response 1 (TIR1) homologs (Dayan et al., 2010; Walsh et al., 2006). However, when present at high concentrations, these compounds, can be herbicidal resulting in deregulation of biochemical and physiological processes in plants, eventually leading to plant damage and death via a three-phase response (Fig. 1.5).

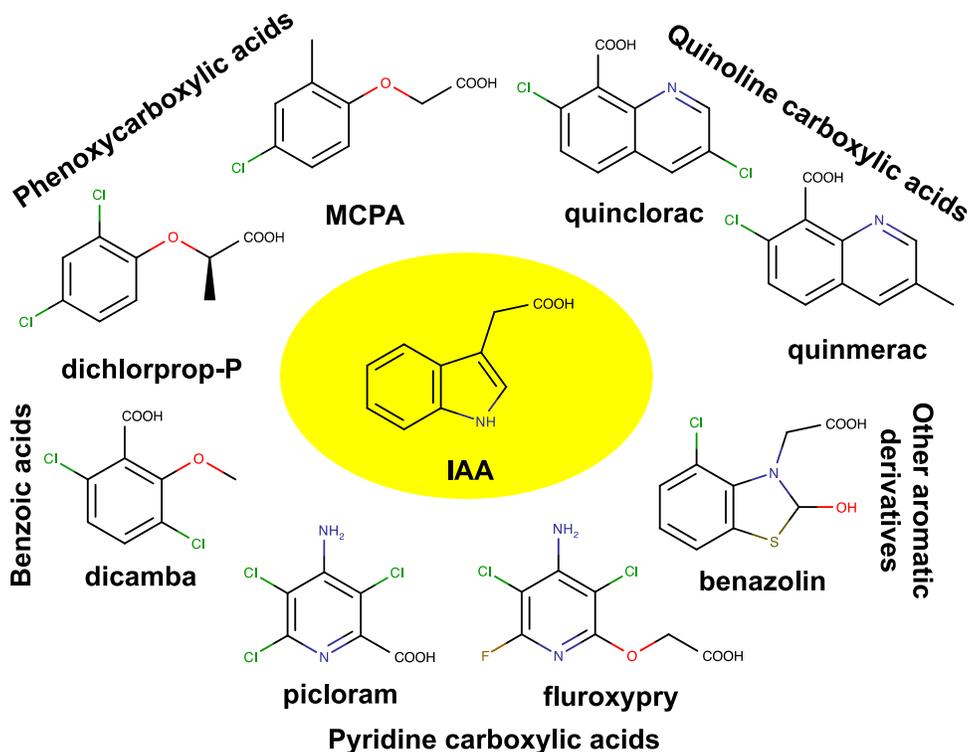


Figure 1.4 Structures of natural indole-3-acetic acid (IAA) and examples of synthetic herbicides from different chemical classes.

Upon auxin herbicide treatment, the three-phase response starts with a stimulation phase, which involves metabolic activation including ATPases, gene expression, ethylene formation, and abscisic acid (ABA) accumulation. At the same time, in the auxinic herbicide-sensitive dicot plants, notably abnormal growth including stem curling, tissue swelling, and leaf epinasty can occur within hours after application of these herbicides. The second phase is inhibition phase, where stunted plant growth (root and shoot growth) and intensification of green leaf pigmentation can occur. Also, other alterations such as stomatal closure, resulting in reduced transpiration, carbon fixation and starch synthesis can occur during this phase. All these reactions will increase formation and accumulation of the reactive oxygen species (ROS) in cells, including both free radicals ($O_2^{\bullet-}$, superoxide radicals; OH^{\bullet} , hydroxyl radical; HO_2^{\bullet} , perhydroxy

radical and RO•, alkoxy radicals) and molecular forms (e.g. H₂O₂, hydrogen peroxide and ¹O₂, singlet oxygen) (Gill and Tuteja, 2010). The last phase is decay phase, due to the formation and accumulation of ROS, leading to membrane damage, vascular system disruption, impaired cell homeostasis, resulting in red discoloration, chlorosis, wilting, necrosis of tissues and ultimately plant death (Grossmann, 2003; Grossmann, 2010).

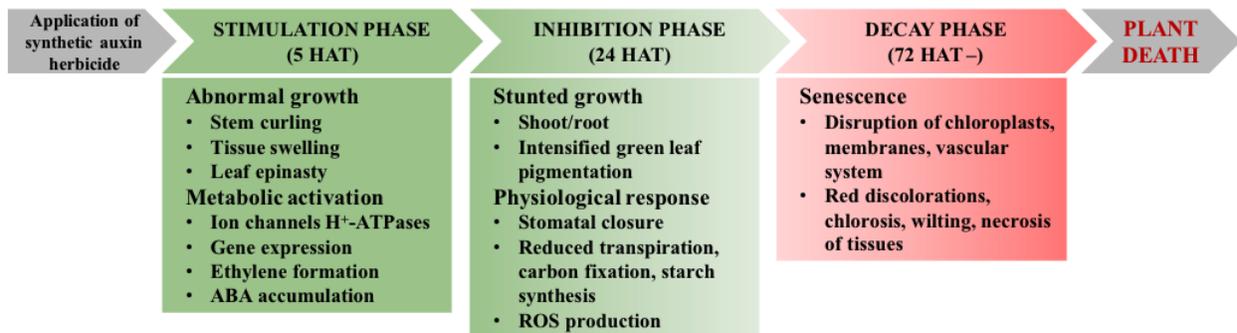


Figure 1.5 Three-phase response to synthetic auxin herbicide in wild biotype of dicot weeds (Modified from Grossmann(2010))

In 2005, after more than 100 years of research efforts across many laboratories worldwide, the molecular target for IAA was discovered (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). A family of six receptors including TIR1, and five auxin-related F-box (AFB) proteins AFB1, AFB2, AFB3, AFB4, and AFB5 were identified as auxin receptors in plants. Auxin plays the role of “molecular glue” of TIR1 protein with the co-receptor Aux/IAA transcription repressor (Gray et al., 2001; Tan et al., 2007). The degradation of Aux/IAA repressors is required for de-repression of the auxin response factors (ARFs) and initiate the downstream biochemical and physiological reactions in the cell. ARFs are also the pre-existing DNA-binding transcriptional activator proteins, including 1-aminocyclopropane-1-carboxylic acid synthase that leads to ethylene and Aux/IAA repressors biosynthesis that are used for feedback inhibition (Guilfoyle, 2007; Hagen and Guilfoyle, 2002).

After unveiling the basis of auxin perception, signaling and gene expression in *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh.), a better understanding of how synthetic auxin herbicides work in plants has been suggested. Several models of the mode of action of auxinic herbicides have been proposed (Grossmann, 2010; Jugulam et al., 2011; Tan et al., 2007), which depict the chronology of events after IAA or synthetic auxin herbicide (e.g. 2,4-D) application (summarized in Fig. 1.6). Briefly, when IAA or 2,4-D reach the apoplast of a plant cell (in the case of IAA, by de novo synthesis or release from stored forms; whereas for 2,4-D, by herbicide application and/or phloem transportation), perception of these molecules by auxin-binding protein 1 (ABP1) in cell membrane, causes rapid cascade of events in cytoplasm including proton pumping, K^+ channel activation, cell wall loosening, and cell expansion/division. At the same time, the IAA and 2,4-D can be actively transported into the cell by carrier proteins. In the cytoplasm, the IAA or 2,4-D is recognized by Skp, Cullin, F-box containing complex ($SCF^{TIR1/AFB}$), with the co-receptor Aux/IAA that is also the repressor protein of auxin responding factors (ARFs). This results in the formation of a “sandwich” of $SCF^{TIR1/AFB}$ protein complex and Aux/IAA, which is “glued” together by the IAA or 2,4-D. This then can lead to ubiquitination of the Aux/IAA protein and finally degraded by 26S proteasome. The degradation of Aux/IAA protein removes the repression effect, which activates the ARFs and rapidly increases the auxin-responsive gene expression for further biochemical and physiological responses (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Gray et al., 2001; Grossmann, 2010; Hagen and Guilfoyle, 2002; Jugulam et al., 2011; Kepinski and Leyser, 2005; Tan et al., 2007).

When the concentration of IAA increases in the cell, biosynthesis of Aux/IAA repressor are induced at the same time to repress the ARFs to deactivate the IAA induced gene expression

(Jugulam et al., 2011; Kelley and Riechers, 2007; Staswick et al., 2005). In addition, GH3-mediated conjugation of IAA with amino acids can rapidly decrease the concentration of IAA in the cytoplasm. Therefore, the IAA perception, signaling, and gene expression are balanced to maintain the auxin homeostasis in plant cells (Bajguz and Piotrowska, 2009; Ludwig-Müller, 2011; Petersson et al., 2009; Staswick, 2009).

Synthetic auxin herbicide 2,4-D and others can also be actively transported into plant cells by active transporters and bind to the TIR1/AFB protein on the SCF^{TIR1/AFB} complex and “glue” to the ARF repressor Aux/IAA protein to cause the Aux/IAA ubiquitination and ultimately degradation by 26S proteasomes. However, 2,4-D and other synthetic auxin herbicides are not substrates of GH3 mediated conjugation and cannot be hydroxylated or detoxified rapidly by P450s or other metabolic pathways in sensitive plants. The uncontrolled high concentration of 2,4-D or synthetic auxin herbicides induce the irreversible three-phase responses to synthetic auxin herbicides as described in Fig. 1.5, which eventually lead to plant death (Grossmann, 2010; Jugulam et al., 2011).

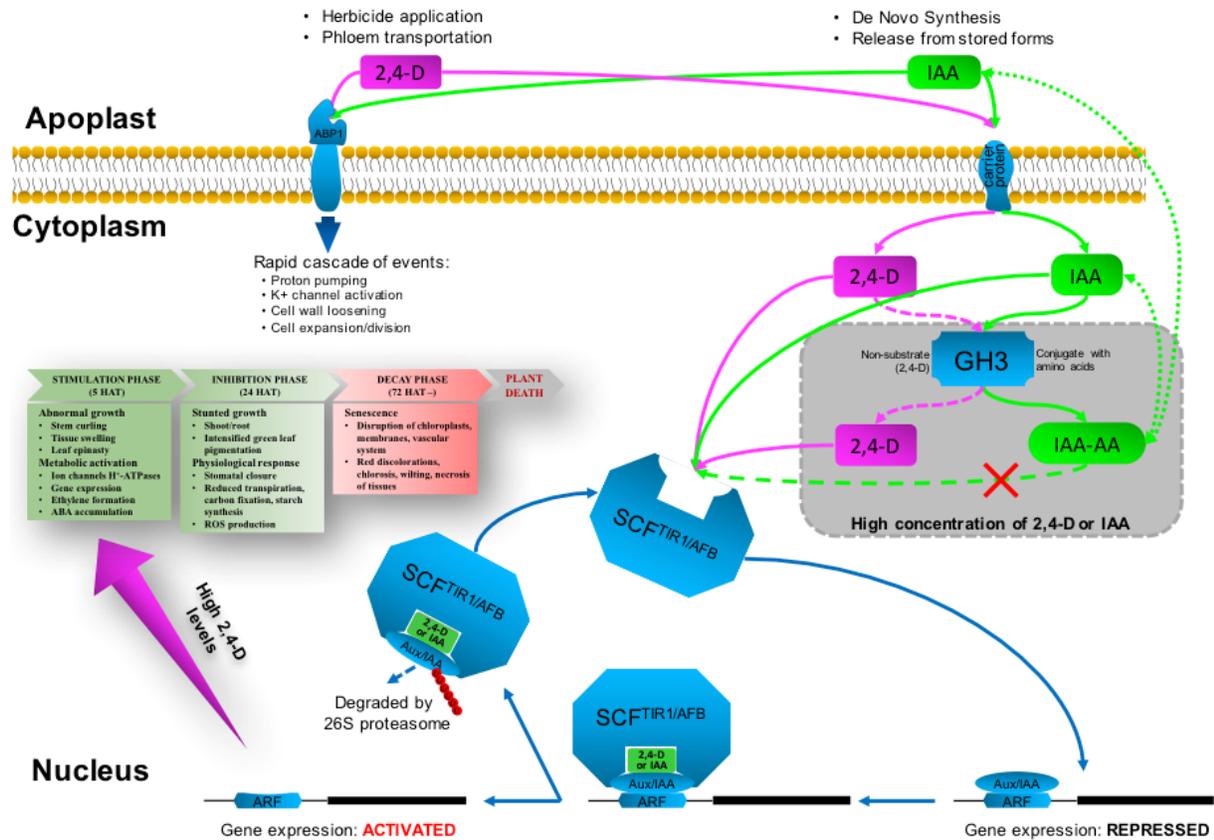


Figure 1.6 Proposed model describing the sequential biochemical and physiological events in cells of sensitive dicot plant after treatment with 2,4-D (a synthetic auxin) and natural auxin indole-3-acetic acid (IAA) (modified from (Grossmann, 2010; Jugulam et al., 2011; Tan et al., 2007)).

1.6 Mechanisms of Synthetic Auxin Herbicide Resistance

To date, 69 unique cases of synthetic auxin herbicide resistance in 36 weed species have been documented (Fig. 1.7) (Heap, 2017), but the thorough investigation of mechanisms of resistance has been reported only in some cases, and the knowledge of molecular basis of synthetic auxin herbicide resistance is still limited.

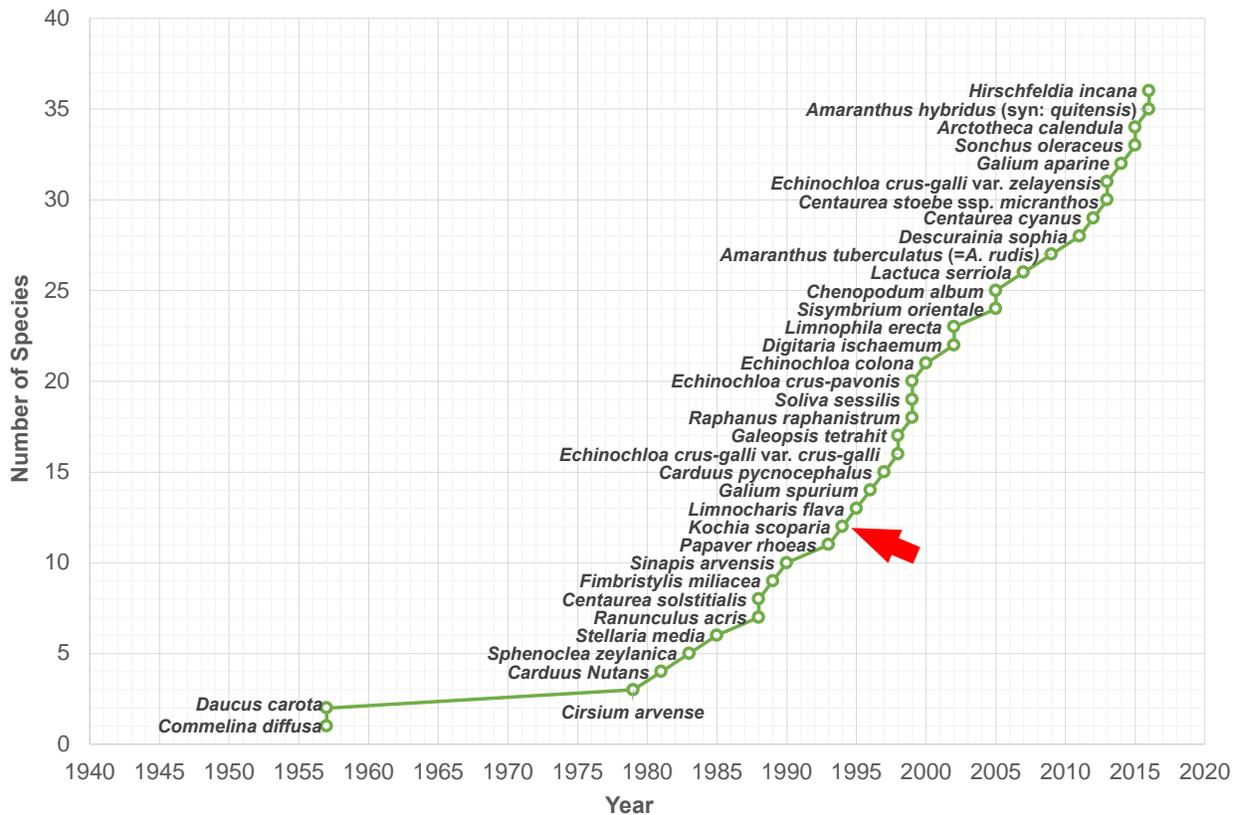


Figure 1.7 Number of weed species (in scientific names) with reported resistance to synthetic auxin herbicides (Adapted and modified from www.weedscience.org (Heap, 2017)), *Kochia scoparia* is highlighted by the arrow.

1.6.1 Barnyardgrass (*Echinochloa crus-galli* (L.) Beauv. var. *crus-galli*) and Other Grasses

Quinclorac, a synthetic auxin herbicide, belongs to quinolone carboxylic acid class, is the auxin herbicide which has activity on certain grass weeds (Grossmann and Kwiatkowski, 2000), probably because of its distinct mode of action on grasses. Some studies suggested that the accumulation of cyanide in cells after quinclorac application may result in plant death (Busi et al., 2017; Grossmann, 2010; Jugulam et al., 2011; Yasuor et al., 2012). So far, five grass weeds were found to have evolved resistance to quinclorac, e.g. barnyardgrass, smooth crabgrass (*Digitaria ischaemum* (Schreb.) Muhl.), gulf cockspur grass (*Echinochloa crusgalli* (L.) Beauv. var. *zelayensis*), gulf cockspur (*Echinochloa crus-pavonis* L.), and junglerice (*Echinochloa colona* (L.) Link) (Heap, 2017). The investigation of quinclorac resistance in barnyardgrass revealed that the resistant biotypes had reduced translocation of quinclorac, but this was not conclusively attributed to the resistance mechanism (Lopez-Martinez et al., 1997; Lovelace et al., 2007). More recently, a 6-10 fold over expression of a GST homologue EcGST1 protein was identified in a quinclorac-resistant barnyardgrass population from China. This over expression may be partially responsible for GST-based metabolism of quinclorac in resistant plants (Li et al., 2013). Also, a quinclorac-resistant smooth crabgrass showed reduced accumulation of ABA and cyanide, and quadrupled β -cyanoalanine synthase activity relative to a sensitive plant. This suggests a higher level of cyanide detoxification and possibly a target-site based resistance may be involved in quinclorac resistance in this smooth crabgrass population (Abdallah et al., 2006). However, more studies are needed to fully uncover both quinclorac mode of action and mechanism of resistance in grasses (Busi et al., 2017).

1.6.2 Wild mustard (*Sinapis arvensis* (L.) Sinar.)

The first case of synthetic auxin herbicide resistant wild mustard was reported in Manitoba, Canada in 1990 (Debreuil et al., 1996; Heap, 2017). A biotype of wild mustard, found in spring barley and wheat cropland, was resistant to 2,4-D, dicamba, dichlorprop, MCPA, mecoprop, and picloram (Debreuil et al., 1996). The dose-response studies revealed high resistance to MCPA, 2,4-D, picloram, and dicamba, relative to sensitive biotypes from the same field (Heap and Morrison, 1992). Synthetic auxin herbicide resistance in wild mustard is not as a result of reduced absorption, translocation, or enhanced metabolism (Penuik et al., 1993). The resistance was attributed to an altered auxin binding site (Deshpande and Hall, 2000). Although, the modification of auxin binding site was not further investigated in wild mustard, reduced ethylene production leading to reduced ACCase expression in resistant biotypes was reported to have played a role in the resistance (Grossmann, 2000; Grossmann, 2010; Hall et al., 1993; Penuik et al., 1993). Also, the role of ABP1 binding affinity to synthetic auxins was investigated in this species and a low affinity-binding site was found in the resistant compared to susceptible wild mustard biotypes. However, other studies did not find a conclusive role of ABP1 in conferring synthetic auxin herbicide resistance in wild mustard (Jugulam and Hall, 2005; Webb and Hall, 1995). More recently, morphological and molecular markers closely-linked to synthetic auxin herbicide resistance were found by amplified fragment length polymorphism (AFLP) analyses in this species (Mithila et al., 2012).

1.6.3 False cleavers (*Galium spurium* (L.) var. *echinospermon*)

In 1996, a quinclorac-resistant false cleaver population was found in a canola and wheat rotation field in central Alberta, Canada (Hall et al., 1998; Heap, 2017). The quinclorac resistance in false cleavers was not because of reduced absorption, translocation or metabolism of this herbicide

(Hall et al., 1998; Van Eerd et al., 2004). However, compared to susceptible biotypes, reduced production of ethylene and ABA was recorded in resistant false cleaver plants (Van Eerd et al., 2005). The results of a genetic study indicated that the quinclorac resistance in this false cleaver population was inherited by a single recessive nuclear gene, and this gene was found not linked to ALS-inhibitor resistance, which is controlled by a single dominant nuclear gene in this multiple herbicide-resistant population (Van Eerd et al., 2004).

1.6.4 Yellow starthistle (*Centaurea solstitialis* (L.) Censo.)

Picloram resistance in yellow starthistle was first reported in the United States in 1988 (Callihan et al., 1990; Heap, 2017). Later, cross-resistance to clopyralid is also found in the same population (Sabba et al., 2003). No difference in absorption, translocation, or metabolism of both picloram and clopyralid was found between the resistant and the susceptible yellow starthistle biotypes, but a 20-fold reduction in ethylene production was observed only in the resistant biotype (Sabba et al., 1998; Valenzuela-Valenzuela et al., 2001). Furthermore, genetic analyses confirmed that the quinclorac resistance is endowed by a recessive nuclear gene in this species (Sabba et al., 2003).

1.6.5 Common hempnettle (*Galeopsis tetrahit* (L.) Gaete)

The first MCPA-resistant common hempnettle population was found in a cereal crop field in Alberta, Canada in 1998, where spring barley and wheat were planted in the rotation (Heap, 2017). This common hempnettle population was also found to be resistant to fluroxypyr, dicamba, and 2,4-D (Beckie et al., 2001). Investigation of the mechanism of resistance in this species revealed that the resistant plants translocated less MCPA out of the treated leaf and also metabolized more in roots relative to a susceptible biotype (Weinberg et al., 2006). Further, it

was reported that MCPA resistance in this common hempnettle population is conferred by at least two nuclear genes with additive effects (Weinberg et al., 2006).

1.6.6 Prickly lettuce (*Lactuca serriola* L.)

In 2007, the first case of prickly lettuce resistance to synthetic auxin herbicide was documented. This prickly lettuce population was found in a cereal field in Washington, USA. The resistant biotype was cross-resistant to 2,4-D, dicamba, and MCPA, and also found resistant to glyphosate (Burke et al., 2009; Heap, 2017; Riar et al., 2011). The resistant biotype absorbed and translocated less 2,4-D compared to the susceptible biotype, but no difference in 2,4-D metabolism between these two biotypes was found (Riar et al., 2011). It was proposed that reduced overstimulation response compared to that in the susceptible biotype after 2,4-D application may be associated with the resistance (Riar et al., 2011). Furthermore, genetic analyses revealed that the 2,4-D resistance was inherited by a single codominant gene in this species (Riar et al., 2011).

1.6.7 Corn poppy (*Papaver rhoeas* L.)

Corn poppy is a major weed impacting cereal production in Europe. In 1993, the first case of 2,4-D-resistant corn poppy was reported from a wheat field in Spain (Cirujeda et al., 2000; Heap, 2017). This population was also resistant to tribenuron, which is an ALS-inhibitor (Cirujeda Ranzenberger, 2001). Later in 1998, two other 2,4-D-resistant corn poppy populations were reported in Italy, one of them is also resistant to two ALS-inhibitors including iodosulfuron, and tribenuron (Heap, 2017). More recently, other corn poppy populations were identified resistant to 2,4-D, dicamba and aminopyralid again, in Spain (Rey-Caballero et al., 2016). Also, a population of corn poppy from France was reported to have evolved resistance to 2,4-D, MCPA, iodosulfuron, mesosulfuron and tribenuron (Délye et al., 2016; Heap, 2017). With the spread of

multiple herbicide resistance, corn poppy is threatening cereal production, especially in southern Europe (Busi et al., 2017). Reduced translocation of 2,4-D resulting in less ethylene production has been attributed to 2,4-D resistance in one of the above populations (Rey-Caballero et al., 2016); whereas, enhanced metabolism by P450s conferring resistance was reported in two other populations (Torra et al., 2017).

1.6.8 Wild radish (*Raphanus raphanistrum* L.)

Wild radish is one of the major problem weeds in Australia (Jones et al., 2005). Evolution of resistance to four modes of action including synthetic auxin herbicides, ALS-, EPSPS-, and carotenoid biosynthesis-inhibitors (WSSA group 11) is a challenge for sustained crop production (Busi et al., 2017; Heap, 2017). First two cases of 2,4-D-resistant wild radish were reported in 1999 (Heap, 2017; Walsh et al., 2004). Subsequent surveys of herbicide-resistant wild radish in western Australian wheatbelt were carried out in 2003, 2010 and 2015, and it was found that 2,4-D resistance had increased from 60% in 2003 to 74% in 2010 and maintained at the same level in 2015 (Owen et al., 2015; Walsh et al., 2007). Reduced translocation of 2,4-D, which could be due to loss of function of an ATP-binding cassette subfamily B (ABCB)-type long-distance auxin efflux transporter, was attributed to 2,4-D resistance in two wild radish populations. The genetic analyses of these populations revealed a nuclear inherited incompletely dominant gene that controls the resistance to 2,4-D (Busi et al., 2017; Busi and Powles, 2017). Similar results were reported in other 2,4-D and MCPA-resistant wild radish populations (Goggin et al., 2016; Jugulam et al., 2013). Furthermore, a difference in auxin perception and/or signal transduction among resistant biotypes was also reported. A genome-wide transcriptomics study of auxin-induced transcriptional repressors and defense genes in wild radish is being conducted by

Australian scientists, which may shed new lights regarding the precise mechanism of synthetic auxin herbicide resistance in wild radish (Busi et al., 2017).

1.6.9 Common waterhemp (*Amaranthus tuberculatus* (L.) Moq.)

Common waterhemp is one of the top five economically important weeds in the United States, especially the rapid and wide spread multiple herbicide resistance is challenging the crop production in many Midwestern states. The first case of 2,4-D-resistant common waterhemp was reported in Nebraska, USA, in 2009 (Bernards et al., 2012). This population is also resistant to aminopyralid, picloram, atrazine, chlorimuron, and imazethapyr (Heap, 2017). In 2016, a common waterhemp was reported in Illinois, USA with resistance to five different modes of action of herbicides, e.g. ALS-, PSII-, PPO-, hydroxyphenylpyruvate dioxygenase (HPPD, WSSA group 27)-inhibitors, and synthetic auxin herbicides (Heap, 2017). Rapid metabolism of 2,4-D via cytochrome P450s activity was found to confer resistance in common waterhemp population from Nebraska, USA (Figueiredo et al., 2017).

1.6.10 Kochia (*Kochia scoparia* (L.) Schrad.)

Among several synthetic auxins, dicamba is found to be most effective on kochia control. Nonetheless, dicamba-resistant kochia was first reported in 1990's in Montana, North Dakota, Idaho, and Colorado, USA (Heap, 2017). Currently, dicamba-resistant kochia is commonly found, especially in wheat-fallow fields in Colorado and Kansas. The physiological, biochemical, and molecular basis for dicamba resistance in kochia from Montana and Colorado has been studied extensively (Cranston et al., 2001; Kern et al., 2005; Pettinga et al., 2017). In the Montana population, no difference in absorption, translocation or metabolism of dicamba was detected between dicamba-resistant and -susceptible biotypes (Cranston et al., 2001). The molecular study indicated several auxin-related transcripts up- or down-regulated upon dicamba

treatment. However, if these genes are directly involved in dicamba resistance mechanism or just auxin-related downstream response remains unknown in this kochia population (Grossmann, 2010; Kern et al., 2005; Zhang and Riechers, 2008). Recently, it was reported that reduced translocation of dicamba contributes to the resistance in a Colorado population (Pettinga et al., 2017). Additionally, an up-regulation of chalcone synthase gene (*CHS*), resulting in over production of the flavonols quercetin and kaempferol was discovered that can compete with dicamba for intercellular movement and vascular loading via ABCB-type membrane transporters, leading to reduced dicamba translocation in the CO kochia population (Pettinga et al., 2017). Furthermore, in the same kochia population, a double mutation in auxin co-receptor gene *Aux/IAA* was identified, which confer low dicamba affinity in Aux/IAA protein complex and thus, kochia plants can cope with high level of dicamba in cells (LeClere et al., 2018).

1.7 Summary

Management of kochia is a major problem in the Great Plains of North America, especially, with rapid spread of dicamba resistance after the evolution and increase in glyphosate and ALS-inhibitor resistant populations in this region (Stahlman et al., 2015). The precise mechanism of dicamba resistance in weeds, including kochia is still not completely known. It is important to investigate the underlying mechanism(s) of dicamba resistance in kochia to formulate best management strategies for its control (Busi et al., 2017; Délye et al., 2013). It is also essential to examine the inheritance and genetic basis of dicamba-resistant trait in kochia, which will help understand the evolutionary dynamics and possible spread of resistance and importantly, to recommend effective and sustainable weed management approaches (Délye et al., 2013; Varanasi et al., 2016; Zheng and Hall, 2001). Also, understanding the influence of climate factors on the efficacy of herbicides, such as dicamba on kochia, will also help to provide recommendations for effective use of herbicides and slow down the development of herbicide resistance (Busi and Powles, 2009), especially with the emerging climate fluctuations across the globe (Bailey, 2004; Millar et al., 2007). Overall, it is important to develop feasible approaches to include in “toolbox” for kochia management, especially dicamba-resistant kochia (Soltani et al., 2016; Soltani et al., 2017).

Thus, the overall goal of this dissertation was to investigate the mechanisms and genetic basis of dicamba resistance in kochia. Furthermore, several management options to control dicamba-resistant kochia were also investigated. The specific objectives of this thesis include:

Chapter 2: 1) determine the level of dicamba resistance in kochia; 2) investigate the physiological basis of dicamba resistance in kochia, including dicamba absorption, translocation,

and metabolism; and 3) investigate the biochemical and molecular basis of dicamba resistance in kochia.

Chapter 3: 1) investigate the inheritance of dicamba resistance in dicamba-resistant kochia populations collected from different geographical regions; 2) determine possible linkage of this trait with glyphosate resistance in populations from Kansas.

Chapter 4: 1) evaluate the effect of temperature on the efficacy of dicamba in kochia control; 2) examine the efficacy of preemergence application of dicamba on dicamba-resistant kochia; and 3) determine the efficacy of dicamba and glyphosate combinations (tank-mixes) on dicamba and glyphosate-resistant kochia control.

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Chapter 2 - Investigation of Mechanism(s) of Dicamba Resistance in Kochia

2.1 Abstract

Synthetic auxin herbicides (e.g. 2,4-D, dicamba, picloram, etc.) are widely used to control dicot weeds in cereal crops around the world. Dicamba, in particular is used widely to control Kochia (*Kochia scoparia* (L.) Schrad), one of the most troublesome weeds throughout the Great Plains of North America. Especially, after the wide spread prevalence of glyphosate resistance in kochia populations in this region, dicamba has been an option for the management of this weed. To date, 69 unique cases of synthetic auxin herbicide resistance in 36 weed species have been documented. The first cases of dicamba resistance in kochia was reported in 1990s in MN, ND, ID, CO, and more recently in KS in 2012. However, the mechanism of resistance to dicamba in kochia from KS is still elusive. The objectives of this study were to characterize the non-target-site and target-site resistance mechanisms to dicamba using kochia populations from KS (KSUR) and CO (CSUR) along with known dicamba-susceptible kochia populations from KS (KSUS) and CO (CSUS) for comparison. A series of experiments were conducted with [¹⁴C] dicamba, to determine uptake, translocation and metabolism of dicamba in these kochia populations. Furthermore, presence of any single nucleotide polymorphism (SNP) and over expression of auxin receptor genes (TIR or AFBs), the possible target sites of dicamba was also investigated. The results of these studies revealed two different mechanisms conferring dicamba resistance in KSUR and CSUR kochia. While reduced translocation of the dicamba contributes to the resistance in CSUR kochia, a SNP in one of *TIR1* homologues was identified in several KSUR kochia plants. Although co-segregation of this SNP with dicamba-resistant phenotype needs

further investigation. Overall, the outcome of this research clearly demonstrates kochia populations can evolve non-target-site or target-site resistance or potentially even both to dicamba, in response to different types of dicamba selection pressure.

2.2 Introduction

Dicamba, a benzoic acid synthetic auxin herbicide, also known as Banvel®, Clarity®, etc., is used widely to control dicot weeds in cereal crops around the world. In Kansas, dicamba has become one of the important herbicide options to control kochia (*Kochia scoparia* (L.) Schrad.), an economically important weed, especially following the wide spread incidence of glyphosate resistance in this region. After the initial incidence of dicamba resistance reported in MT, ND, ID and CO in early 1990s (Heap, 2017), more recently evolution of dicamba resistance in kochia has been documented in numerous fields, especially in wheat-fallow in CO and KS (Heap, 2017). A number of previous studies investigated the mechanism of dicamba resistance in kochia (Cranston et al., 2001; Kern et al., 2005; Pettinga et al., 2017). In a MT population, no difference in absorption, translocation or metabolism of dicamba was found between dicamba-resistant and -susceptible biotypes (Cranston et al., 2001). Further investigation using a differential display technique indicated up- or down-regulation of several auxin-related transcripts in response to dicamba treatment. However, if these genes are directly involved in dicamba resistance mechanism or just auxin-related downstream response remains unknown in this kochia population (Kern et al., 2005). Recently, it was reported that reduced translocation of dicamba contributing to the resistance in a kochia population from CO (Pettinga et al., 2017). Additionally, up-regulation of chalcone synthase gene (*CHS*) was discovered, which results in over production of the flavonols quercetin and kaempferol that can compete with dicamba for intercellular movement and vascular loading via ABCB-type membrane transporters, leading to reduced dicamba translocation in this dicamba-resistant kochia population (Pettinga et al., 2017). Furthermore, in this same kochia population, a double mutation in *Aux/IAA*, an auxin co-receptor gene, was also identified, leading to low dicamba affinity in Aux/IAA protein complex, thereby,

kochia plants can cope with the high level of dicamba in cells (LeClere et al., 2017). However, the mechanism of dicamba resistance in kochia from KS is still not clear. The recent research (Pettinga et al., 2017) was also a part of this dissertation and therefore, the objectives of this research were to: 1) investigate the physiological basis of dicamba resistance in kochia from KS and CO, by determining the [^{14}C] dicamba absorption, translocation, and metabolism; and 2) investigate the biochemical and molecular basis of dicamba resistance in kochia from KS.

2.3 Materials and Methods

In 2012, kochia seed were collected from a field in Haskell County, KS, USA (37°29'48.5"N, 100°46'53.0"W) (Brachtenbach, 2015). Kochia plants generated from these seeds were self-pollinated by keeping the plants in isolation and upon maturity seed were harvested separately from each of ten plants. One hundred seedlings were generated separately after harvesting the seed from each of the above 10 plants. When plants reached 10-12 cm height, 50 plants each were treated with a field rate of dicamba (560 g ae ha⁻¹). In response to dicamba treatment, all the progeny of a single plant that were found susceptible to dicamba were selected as dicamba-susceptible kochia (KSUS). The remaining seed harvested from the KSUS mother plant was used in this research. Likewise, all the progeny of single plant that were found resistant to the field rate of dicamba were selected as dicamba-resistant kochia (KSUR). Similarly, the rest of the seed harvested from KSUR mother plant was used in this research. Inbred dicamba-resistant (CSUR, also known as 9425R) and dicamba-susceptible (CSUS, also known as 7710S) kochia lines from CO, derived by single-seed descent for four generations followed by bulk seed production for 13 generations (Howatt et al., 2006; Preston et al., 2009) were also used in this research.

Experiments were conducted in weed science greenhouse attached to the Department of Agronomy at Kansas State University, Manhattan, Kansas, United States. The following greenhouse conditions were maintained: 25/20 °C (day/night, d/n) temperatures, 60 ± 10% relative humidity, and 15/9 h d/n photoperiod supplemented with 120 µmol m⁻² s⁻¹ illumination provided with sodium vapor lamps. The physiological studies were conducted in growth chambers maintained at following conditions: 25/15°C d/n temperature, 60 ± 10% relative humidity, and 15/9 h d/n photoperiod, light was provided by incandescent and fluorescent bulbs delivering 750 µmol m⁻² s⁻¹ photon flux at plant canopy level. Solvents and reagents were used in

the studies in this chapter that are not specified in the context were purchased from Thermo Fisher Scientific (Waltham, MA, USA) or MilliporeSigma Corp. (Burlington, MA, USA).

2.3.1 Dose Response of Dicamba in KSUR, KSUS, CSUR, and CSUS kochia

KSUR, KSUS, CSUR, and CSUS kochia seeds were germinated in trays (25 × 15 × 2.5 cm) filled with commercial potting mixture (Pro-Mix Potting-Mix, Premier Tech Horticulture, Ontario, CA). Individual seedlings at 6-leaf stage were transplanted into plastic pots (6.5 × 6.5 × 9 cm) containing the same type of soil and kept in the same greenhouse as above. When the kochia seedlings were 10-12 cm height, they were treated with dicamba (Clarity[®], BASF Corp., Florham Park, NJ, USA) without AMS at 0, 70, 140, 280, 560 (label recommended field, i.e. 1X dose), 1120, 2240, 4480, and 8960 g ae ha⁻¹.

The above treatments were applied as follows. Herbicides were mixed according to the labels and applied using a bench-type sprayer (Research Track Sprayer, De Vries Manufacturing, Hollandale, MN, USA) equipped with a single moving even flat-fan nozzle tip (8002E TeeJet tip, Spraying Systems Co., Wheaton, IL, USA) delivering 187 L ha⁻¹ at 207 kPa in a single pass at 4.85 km h⁻¹. At four weeks after herbicide treatment (WAT), glyphosate- and dicamba-induced visual injury was rated based on composite visual estimation of growth inhibition, epinasty (downward curling of plant parts), necrosis, and plant vigor on a scale of 0 (no effect) to 100 (plant death). Plant were clipped off at soil level at 4 WAT and individual plants were placed in separate paper sacks. Dry biomass data was obtained by weighing after oven dried at 60 °C for 72 h.

2.3.2 Absorption and Translocation of [¹⁴C] Dicamba

Prior to conducting the absorption and translocation experiments, a preliminary study was conducted to test whether absorption or translocation of [¹⁴C] dicamba in KSUR, CSUR, KSUS,

and CSUS kochia would be affected by spraying plants with formulated dicamba (Clarity® herbicide, BASF Corp., Florham Park, NJ, USA) using the method described by Perez-Jones et al. (2007). Briefly, two newly expanded leaves were marked and wrapped with small pieces of aluminum foil on kochia at height of 10-12 cm, then the plants were sprayed with 560 g ha⁻¹ of dicamba using a bench-type sprayer (Research Track Sprayer, De Vries Manufacturing, Hollandale, MN, USA) equipped with a single moving flat-fan nozzle tip (80015E TeeJet tip, Spraying Systems Co., Wheaton, IL, USA) delivering 187 L ha⁻¹ at 222 kPa in a single pass at 3.21 km h⁻¹. After the herbicide droplets dried (~30 min), the aluminum foil was removed. Two newly expanded leaves were marked on another set of same size kochia seedlings without the 560 g ha⁻¹ of dicamba application. On both sets of plants, the absorption and translocation of [¹⁴C] dicamba were tested using the method described below. Results (data not shown) showed that neither absorption nor translocation of [¹⁴C] dicamba in both dicamba-resistant (DR) and dicamba-susceptible (DS) kochia was affected by spray of dicamba at 560 g ha⁻¹. Hence, in all other experiments using [¹⁴C] dicamba, the plants were not pre-sprayed with formulated dicamba.

Preliminary testing of [¹⁴C] dicamba translocation in kochia also revealed only less than 5% of dicamba translocated to roots at 72 hours after treatments (HAT) and majority (88 to 95%) was recovered from the aboveground parts of kochia. Hence, the amount of [¹⁴C] dicamba translocated to roots was not measured in subsequent experiments.

The absorption and translocation experiments were conducted according to the method that reported by Ou et al. (2016), which is also summarized in Fig. 2.1. A working stock solution of 1 mL of [¹⁴C] dicamba (equal to 560 g of dicamba in a carrier volume of 187 L) with 0.33 kBq μL⁻¹ of radioactivity was prepared by mixing 29.3 μL of dicamba-(ring-UL-¹⁴C) ethanol solution

(11.4 kBq μL^{-1} , specific activity: 2.87 kBq μg^{-1} , BASF Corp., Florham Park, NJ, USA), 6.4 μL of Clarity herbicide (BASF Corp., Florham Park, NJ, USA) and 964.3 μL of water. Ten μL of [^{14}C] dicamba working solution was applied on the upper surface of two newly expanded leaves (5 μL per leaf) for each plant using a Wiretrol[®] capillary syringe (10 μL , Drummond Scientific Co., Broomall, PA, USA). Thirty minutes later, plants were returned to growth chamber. Plants were harvested at 24, 48, 72, 96, 168, and 336 HAT, and then dissected into treated leaf (TL), tissue above the treated leaf (ATL), and tissue below the treated leaf (BTL). After the TL was washed twice in 20-mL scintillation vials for 1 min using 5 mL of 10% (v/v) ethanol aqueous solution with 0.5% of Tween-20) at each time, 15 mL of Ecolite(+) (MP Biomedicals, LLC, Santa Ana, CA, USA) was added in each vial and the radioactivity in TL rinsates was measured using liquid scintillation spectrometry (Beckman Coulter LS6500 Multipurpose Scintillation Counter, Beckman Coulter, Inc. Brea, CA, USA). Dissected plant sections were dried at 60 °C for 72 h and radioactivity in TL, ATL, and BTL was quantified by liquid scintillation spectrometry (LSS) after combusting for three minutes using a biological oxidizer (OX-501, RJ Harvey Instrument, New York, NY, USA). Four replicates were included in each treatment, and the experiment was repeated twice in time.

The calculation of percentage of [^{14}C] absorption, and translocation was done using following formulae. Percentage of absorption= $(R_{\text{applied}}-R_{\text{rinsate}})/R_{\text{applied}}\times 100$, percentage of translocation= $100-R_{\text{TL}}/(R_{\text{applied}}-R_{\text{rinsate}})\times 100$, percentage in ATL= $R_{\text{ATL}}/(R_{\text{applied}}-R_{\text{rinsate}})\times 100$, percentage in TL= $R_{\text{TL}}/(R_{\text{applied}}-R_{\text{rinsate}})\times 100$, and percentage in BTL= $R_{\text{BTL}}/(R_{\text{applied}}-R_{\text{rinsate}})\times 100$. Where, R_{applied} is total amount of radioactivity applied on the plant; R_{rinsate} is the radioactivity recovered in leaf rinsates; R_{ATL} is the radioactivity recovered in tissue above the treated leaf; R_{TL}

is the radioactivity recovered in the treated leaf; and R_{BTL} is the radioactivity recovered in tissue below the treated leaf.

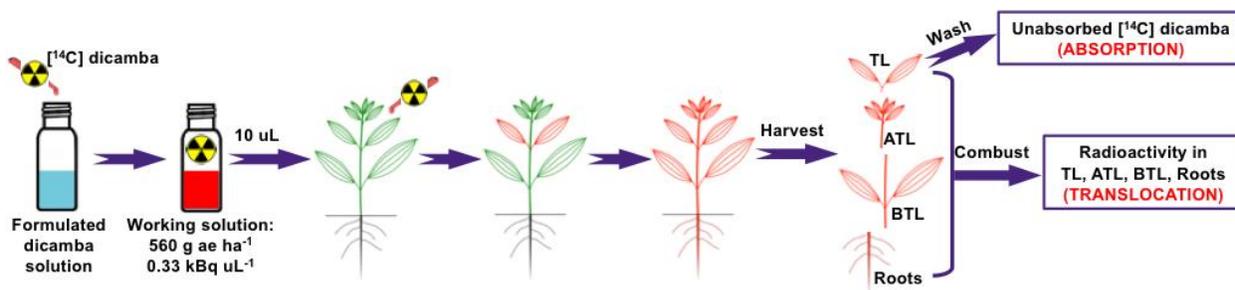


Figure 2.1 Illustration of steps followed in $[^{14}\text{C}]$ dicamba absorption and translocation studies in kochia

2.3.3 Metabolism of $[^{14}\text{C}]$ Dicamba

To determine the metabolism of dicamba, kochia plants were treated with $[^{14}\text{C}]$ dicamba as described above for absorption or translocation study, except the working stock solution was newly mixed with $0.5 \text{ kBq } \mu\text{L}^{-1}$ of radioactivity (Fig. 2.2). The samples were harvest at 24, 48, 72, 96, 168, and 336 HAT according to the method that reported by Godar et al. (2015). At the time of plant harvest, the TLs were dissected and washed as described above in absorption and translocation study. The aboveground part of the plant combining with the washed TL was then frozen in liquid nitrogen and homogenized with a mortar and pestle. The plant powder was extracted with 15 mL of 90% acetone aqueous solution at 4°C for 24 h. Samples were centrifuged at 10,000 g for 10 min at 4°C in centrifuge (Sorvall RC-5B Refrigerated Superspeed Centrifuge, DuPont Company, Newtown, CT, USA). Supernatant was concentrated down to approximately 500 μL at 45°C for 2 h using a rotary evaporator (Centrivap, Labconco, Kansas City, MO, USA). The concentrated samples were transferred to microcentrifuge tubes and centrifuged at 10,000 g for 10 min in centrifuge (Sorvall Legend Micro 21 Microcentrifuge,

Thermo Fisher Scientific, Waltham, MA, USA) and transferred into new tubes. Concentration of radioactivity in each sample was measured by LSS prior to high performance liquid chromatography (HPLC) analysis and samples were adjusted to $1.0 \text{ Bq } \mu\text{L}^{-1}$ by adding 50% (v/v) acetonitrile aqueous solution. All samples and a [^{14}C] dicamba parent sample in acetonitrile with $1.0 \text{ Bq } \mu\text{L}^{-1}$ of radioactivity were analyzed by reverse-phase HPLC (System Gold, Beckman Coulter, Pasadena, CA, USA) using a Zorbax SB-C18 column (250×4.6 mm, 5 μm particle size; Agilent Technologies, Santa Clara, CA, USA) at a flow rate of 1 mL min^{-1} with eluent A (water with 0.1% trifluoroacetic acid, TFA) and eluent B (acetonitrile with 0.1% TFA). The following elution method was used: 0 to 1 min, 0 to 20% (of eluent B) linear gradient; 1 to 3 min, 20 to 40% linear gradient; 3 to 7 min, 40 to 60% linear gradient; 7 to 19 min, 70 to 90% linear gradient; 19 to 21 min, 90 to 40% linear gradient; 21 to 23 min, 40 to 0% linear gradient; 23 to 25 min, 0% isocratic hold to re-equilibrate the column for the next sample injection (25 min total). A radioflow detector (EG &G Berthold, LB 509, Bad Wildbad, Germany) was used to detect radioactivity of the samples after mixing with Ultima-Flo M cocktail (PerkinElmer, Waltham, MA, USA). Via this HPLC method, the [^{14}C] dicamba parent compound exhibited a retention time of 10.9 min. The amount of [^{14}C] dicamba parent was quantified as a percentage of total detected radioactivity based on peak area in the chromatograph. Treatments were replicated four times and the experiment was repeated.

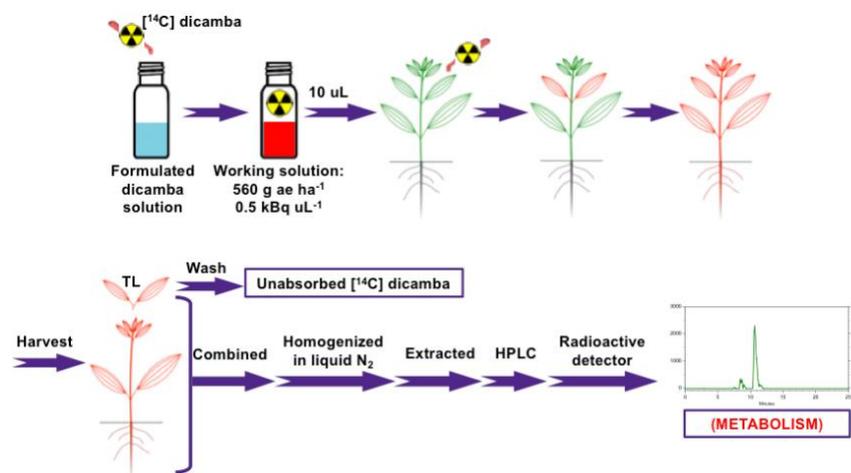


Figure 2.2 Illustration of steps followed in $[^{14}\text{C}]$ dicamba metabolism study

In the absorption, translocation, and metabolism studies, a complete randomized experimental design was used. Analysis of variance was conducted in Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA).

2.3.4 Phosphor Image Analysis

A separate working stock solution of $[^{14}\text{C}]$ dicamba with $3.3 \text{ kBq } \mu\text{L}^{-1}$ of radioactivity and equal to 560 g of dicamba in a carrier volume of 187 L was prepared (Fig. 2.3). Kochia seeds were germinated in potting mixture (Pro-Mix Potting-Mix, Premier Tech Horticulture, Ontario, CA). Individual seedlings from each population at 2 to 3 cm height were transplanted into plastic pots ($6.5 \times 6.5 \times 9 \text{ cm}$) that filled up with silica sand (Granusil® Handy Sand, Fairmount Santrol, Sugar Land, TX, USA) that has rinsed in 0.1% (w/v) of fertilizer (Miricle-Gro water soluble all-purpose plant food, N:P:K of 24:8:16, Scotts Miracle-Gro Products Inc. Marysville, OH, USA), and kept in growth chamber maintained at the same settings as described above. When the kochia seedlings were 6-8 cm height, they were treated with $1 \mu\text{L}$ of the working solution on a newly expanded leaf. At 48, 72, and 96 HAT, the treated plants were gently uprooted and the roots were washed with water carefully to remove soil particles. Subsequently, the whole plant

was washed twice with 10 mL of 10% (v/v) ethanol aqueous solution that contains 0.5% of Tween-20, and then pressed using a plant press (Lacey et al., 2001) and dried at 60°C for 72 h. The pressed kochia plants were exposed to BAS-IP MS 2040 E Multipurpose Standard Storage Phosphor Screen (GE Healthcare Life Sciences, Pittsburgh, PA, USA) for 24 h, and the screen was read using Bio-Rad Molecular Imager FX (Bio-Rad Laboratories, Inc. Hercules, CA, USA). The phosphor images were processed using Quantity One software (v4.6.9, Bio-Rad Laboratories, Hercules, CA, USA). The RGB images used for visualization were processed in GNU Image Manipulation Program 2.8.20 (GIMP development team, <https://www.gimp.org>). Four replicates were included in each treatment using complete a randomized experimental design, experiment was repeated twice.

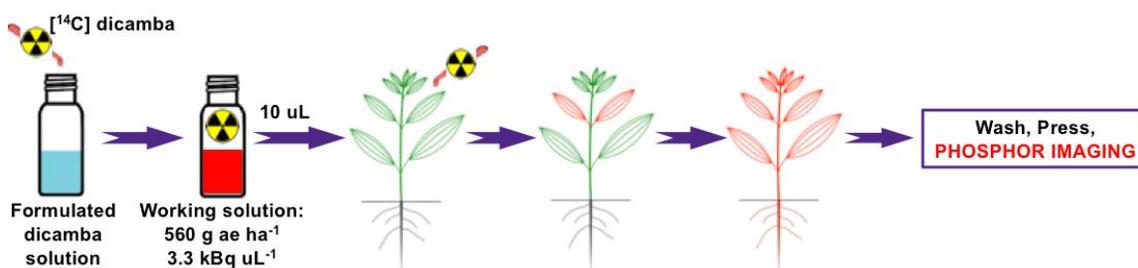


Figure 2.3 Illustration of steps followed in phosphor imaging analysis using [¹⁴C] dicamba

2.3.5 RNA Extraction, cDNA Synthesis and *TIR1* Homologs Expression and Sequencing

Fresh plant tissue from KSUR and KSUS kochia plants (non-treated with dicamba) were collected and flash frozen in liquid nitrogen and stored at -80°C for RNA isolation. The frozen tissue was homogenized in liquid nitrogen using a pre-chilled mortar and pestle. One hundred mg of homogenized tissue was transferred into a 2.5 mL microcentrifuge tube. Total RNA was isolated using Invitrogen™ TRIzol™ RNA extraction kit (Thermo Fisher Scientific, Waltham, MA, USA). In brief, 1 mL of TRIzol™ reagent was added into the 100 mg homogenized plant

tissue. After 5 minutes of incubation at room temperature, 0.2 mL of chloroform was added, and the mix was vortexed briefly. The organic phase and aqueous phase were separated using centrifugation at 12,000 g for 15 min at 4°C. The aqueous phase was transferred into a fresh 2.5 mL microcentrifuge tube and 0.5 isopropanol was added. After incubating for 10 min, the sample was centrifuged at 12,000 g for another 10 min at 4°C, the supernatant was discarded. The pellet of RNA was resuspended in 1 mL of 75% ethanol by vortexing and centrifuged at 7,500g for 5 min at 4°C. After discarding the supernatant, the pellet was air dried for 5-10 min and the RNA was eluted in 40 µL of RNase-free water. The quality and quantity of total RNA was determined using agarose gel (1%) electrophoresis and spectrophotometer (NanoDrop 1000, Thermo Scientific, Waltham, MA, USA) and RNA was store at -80°C.

The RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for cDNA synthesis. The protocol as described in the kit was followed. Briefly, 1 µg of total RNA was incubated at 37°C for 30 min with 1 µL of DNase I enzyme and the 10X reaction buffer with MgCl₂ after normalizing to 10 µL using RNase-free water in a RNase-free tube to remove any genomic DNA. After incubating for another 10 min at 65°C with 1 µL of 50 mM EDTA, 1 µL of Oligo (dT) primer was added and incubated at 65°C for 5 minutes. The reaction was chilled on ice, after that the following reagents were added in order: 4 µL of 5X reaction buffer (contains 250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl₂, and 50 mM dithiothreitol (DTT)), 1 µL of RiboLock RNase Inhibitor (20 U/µL), 2 µL of 10 mM dNTP Mix, and 1 µL of RevertAid M-MuLV RT (200U/µL). The reaction was mixed gently and incubated at 42°C for 60 min and terminated by incubating at 70°C for 5 min. The cDNA product was diluted in 1:5 ratio for gene expression and sequencing studies. The quality of cDNA was tested by PCR reaction using *EPSPS* gene. The PCR reaction was prepared with

mixing 2.5 μ L of forward primer (5 μ M, 5' -GGCCAAAAGGGCAATCGTGGAG-3') and 2.5 μ L of reverse primer (5 μ M, 5'-CATTGCCGTTCCCGCGTTTCC-3') of *EPSPS* gene (Varanasi et al., 2015), 2.5 μ L of cDNA, 12.5 μ L of PCR master mix (Promega, Madison, WI, USA), and 5 μ L of nuclease-free water. The PCR conditions were at 95°C for 3 min of initial denaturing; 40 cycles of 95°C for 10 s of denaturing, 60°C for 30 s of annealing, and 72°C for 30 s extension; and 72°C for 4 min of final extension. The quality of PCR product was determined using agarose gel (1%) electrophoresis.

For *TIRI* homologs expression study, the qPCR reaction mix consisted of 8 μ L of SYBR Green mastermix (Bio-Rad Inc., Hercules, CA, USA), 2 μ L each of forward and reverse primers (5 μ M), and 20 ng cDNA to make the total reaction volume of 14 μ L. *TIRI* homologous genes expression was normalized using *ALS* gene (forward primer: 5'-CCGTTCTCCCTTTCCTCTTT-3' and reverse primer: 5'-GGAAGGTGGTGAGTGGATTTTG-3) as the reference (Varanasi et al., 2015; Wiersma et al., 2015). qPCR was performed in an Applied Biosystems StepOnePlus Real-Time PCR Systems (Thermo Fisher Scientific, Waltham, MA, USA) at PCR conditions were 95°C for 15 min and 40 cycles of 95°C for 30 s and 60°C for 1 min (Varanasi et al., 2015). A melt curve profile was included following the thermal cycling protocol to determine the specificity (no primer dimers, no genomic DNA contamination, and no non-specific product) of the qPCR reaction (Varanasi et al., 2015). Primers for *TIRI* homologs are designed in Primer3 (Thornton and Basu, 2011; Untergasser et al., 2012) and their sequences are listed in Table 2.1. The *TIRI* homologous gene copy numbers were determined using the $2^{\Delta C_t}$ method, where C_t is the threshold cycle and ΔC_t is $C_{t(\text{target gene})} - C_{t(\text{ALS})}$ (Gaines et al., 2010; Jugulam et al., 2014). The experiment was repeated three times.

Table 2.1 The sequences of primers for *TIR1* homologs expression analysis

Gene Name	Primer	Sequence (5'-3')	Size of amplicon (bp)
QNA3953	Forward	ATTCGGGATTGTCCGTTTG	142
	Reverse	CGTTTAGTTGTGGCATCTTCCT	
QNA6948	Forward	TGAACAAGGTTTGGTTTCAGTT	87
	Reverse	AAAGCTTCATTGGACATTTGG	
QNA3015	Forward	TTCTCGCTGGCTGTCCTAA	140
	Reverse	GGCACTCATTGTGACTTTGC	
QNA66	Forward	GGAAATCACTCTTGTTTGCTGT	113
	Reverse	CATCATTCACCTCATCCTCAA	

For *TIR1* homologs sequencing study, A PCR was performed in a T100 thermal cycler (Bio-Rad Inc., Hercules, CA, USA) using PCR master mix (Promega, Madison, WI, USA).

Primer sequences are listed in Table 2.2. The 25 μL reaction volume consisted of 12.5 μL of PCR master mix (2X), 2.5 μL of forward primer (5 μM), 2.5 μL of reverse primer (5 μM), 2.5 μL of cDNA, and 5 μL of nuclease-free water. For amplification of the genes, the following PCR conditions were used: 95°C for 3 min, 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min, followed by 72°C for 5 min. The PCR products were run on 1% agarose gel with 1 kbp markers to confirm amplicon size. PCR products were purified using GeneJet PCR purification kit (Thermo Fisher Scientific, Waltham, MA, USA) and quantified using a spectrophotometer (NanoDrop 1000, Thermo Scientific, Waltham, MA, USA). About 10 μL of the purified PCR product (25 ng μL^{-1}) was sequenced using an ABI 3730 DNA analyzer (Life Technologies, Carlsbad, CA, USA).

Table 2.2 The sequences of primers for *TIR1* homologs sequencing

Gene Name	Primer	Sequence (5'-3')	Size of amplicon (bp)
QNA3953	Forward	TGCGGATTTCAATTTGGTGCCTC	1294
	Reverse	TCGCATTGTCGCATACTCCCA	
QNA6948	Forward	TTCATCGGAAACTGCTACGCT	1525
	Reverse	AAAGCTTCTCAACCGGGCAT	
QNA3015	Forward	TGGGCTTGCTTGAATCTCACA	1266
	Reverse	AAAGTGCGAGTCTTCTGAGCTT	
QNA66	Forward	AGGCCTCGTTTCGCTGATTT	1404
	Reverse	CCCACTAACAAACACCTCGACT	

Translation of gene sequences into amino acid sequences was performed using Sequence Manipulation Suite (Stothard, 2000). Nucleotide sequences of *TIR1* homologs and amino acid sequences were aligned using MultAlin software to analyze the presence of any target-site mutation(s) (Corpet, 1988).

2.3.6 Protein Extraction, SDS-PAGE, Native-PAGE, and Electrophoretic Transfer

The above ground plant tissue (0.5 g) from 10-12 cm height KSUR and KSUS kochia was homogenized in liquid nitrogen. Ten mL of extraction buffer (50 mM Tris-HCl, pH 8, 50 mM NaCl, 1mM EDTA, 1mM MgCl₂, 0.038 g PMSF, one tablet of Pierce Protease Inhibitor (Thermo Fisher Scientific, Waltham, MA, USA), and 1g insoluble polyvinyl polypyrrolidone (PVPP)) was added to the homogenized leaf sample. The modified TCA/acetone method was used to extract and purify the total protein (Nakka, 2016; Wang et al., 2006; Wu et al., 2014). In short, homogenates were centrifuged at 4°C, 10 min, 16,000 rpm and supernatant was collected. One mL of trichloroacetic acid (TCA, 100%) was added to the supernatant and incubated for 1 h at 4°C. The supernatant was discarded after being centrifuged (4°C, 10 min, 16000 rpm), and 2 mL of methanol (100%) was added to the pellet and vortexed vigorously for 60 seconds and

centrifuged (4°C, 10 min, 16000 rpm). Supernatant was discarded and 2 mL of 80% acetone aqueous solution was added to the pellet, vortexed and centrifuged (4°C, 10 min, 16000 rpm). Pellet was air dried to remove the remaining acetone and 2 ml phenol (equilibrated with Tris-HCl; pH 8.0) was added and vortexed at high speed for 30-60s and centrifuged (4°C, 10 min, 16000 rpm) and the supernatant was collected. Proteins were precipitated by adding 2 mL of ammonium acetate (0.1 M in methanol) to the supernatant and incubated overnight at -20°C. Next, the sample was centrifuged (4°C, 10 min, 16000 rpm) and the supernatant was discarded. Pellet was washed with methanol (100%) followed by acetone (80%) and finally air dried. Dried samples were resuspended in 200 µL SDS-sample buffer and the protein concentration in the extract was determined using the PierceTM modified Lowry protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Native total protein extraction was performed using the method as described by Laing and Christeller (2004). In brief, frozen freshly collected kochia leaf tissue (1 g) was mixed with 0.5 g of SiO₂ (acid washed) and ground into powder using a liquid nitrogen chilled mortar and pestle. After liquid N₂ was evaporated and before the tissue started to thaw, 5 mL of ice-cold extraction buffer (0.2 M 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.0; 5% (w/w) PVPP (weigh out, add to mixture, and allow to hydrate 1 h before use); 1% (v/v) Triton X-100; 10% (v/v) glycerol; and Pierce Protease Inhibitor (one tablet in 20 mL of buffer); and 2 mM dithiothreitol (DTT) was added before use) was added and the samples were gently ground. Sample was centrifuged at 20,000 g for 10 min at 4°C. The supernatants were collected into a new tube and centrifuged again at 30,000 g for 30 min at 4°C. The protein content was tested using the RED 660TM Protein Assay kit (Geno Technology Inc., St. Louis, MO, USA).

SDS-PAGE was performed using the Mini-PROTEAN® Tetra Cell system (Bio-Rad Inc., Hercules, CA, USA) and the total protein was resolved by electrophoresis on an 11% polyacrylamide gel (90 min, 120 V, room temperature). The Native-PAGE was conducted using the same system. The total native protein was incubated with 1 kBq of [¹⁴C] labelled dicamba before loading the sample on the 11% polyacrylamide gel (300 min, 50 V, 4°C), and then transferred to polyvinylidene difluoride (PVDF) membrane (0.45 µm pore size, MilliporeSigma, Burlington, MA, USA) at 30 V for 12 h. The membrane was detected using the phosphor imaging system as described above.

2.4 Results and Discussion

2.4.1 Dose Response of Dicamba in KSUR, KSUS, CSUR, and CSUS kochia

The results of dicamba dose response of KSUR and KSUS (Fig. 2.4 and 2.5) suggested that the KSUR kochia were resistant to dicamba at 560 g ae ha⁻¹. For instance, ED₅₀ (effective dose for 50% control of kochia) and GR₅₀ (effective dose for 50% biomass reduction) of dicamba for KSUR kochia were 1259 and 2410 g ae ha⁻¹ (Table 2.3), respectively, which were higher than 560 g ae ha⁻¹. On the other hand, the values of ED₅₀ and GR₅₀ of dicamba for KSUS kochia were significantly lower at 72 and 99 g ae ha⁻¹ (Table 2.3), respectively. Based on ED₅₀ or GR₅₀ estimates, the KSUR kochia was found to be 18 or 24 times more resistant to dicamba than KSUS (Table 4.4) based, respectively.

Similarly, the results of dicamba dose response CUSR and CSUS (Fig. 2.6 and 2.7) suggested that the CSUR kochia were resistant to dicamba too. For instance, ED₅₀ (effective dose for 50% control of kochia) and GR₅₀ (effective dose for 50% biomass reduction) of dicamba for CSUR kochia were both around 970 g ae ha⁻¹ (Table 2.3), respectively, which were higher than 560 g ae ha⁻¹. On the other hand, the values of ED₅₀ and GR₅₀ of dicamba for CSUS kochia were significantly lower at 99 and 182 g ae ha⁻¹ (Table 2.3), respectively. Based on ED₅₀ or GR₅₀ estimates, the KSUR kochia was found to be 10 or 5 times more resistant to dicamba than KSUS (Table 2.3) based, respectively.

Table 2.3 Estimated values of ED₅₀ and GR₅₀ of dicamba in KSUR, KSUS, CSUR, and CSUS kochia using the nonlinear regression analysis of four parameter log-logistic model*.

Kochia	ED ₅₀ [#]	GR ₅₀ [#]
	-----g ae ha ⁻¹ -----	
KSUR	1259(310)	2410(397)
KSUS	72(4)	99(13)
Resistance indices ⁺	18(4)	24(5)
CSUR	974(56)	971(127)
CSUS	99 (2)	182 (24)
Resistance indices ⁺	10(1)	5(1)

* model: $Y=C+(D-C)/(1+\exp[b(\log(x)-\log(I_{50}))])$; ED₅₀ (effective dose for 50% control of kochia) and GR₅₀ (effective dose for 50% biomass reduction) values were estimated using the number of plants data and dry biomass data, respectively. [#] Values in parenthesis are standard error. ⁺ resistant level of GDR kochia population comparing to GDS population using ED₅₀ or GR₅₀ values.

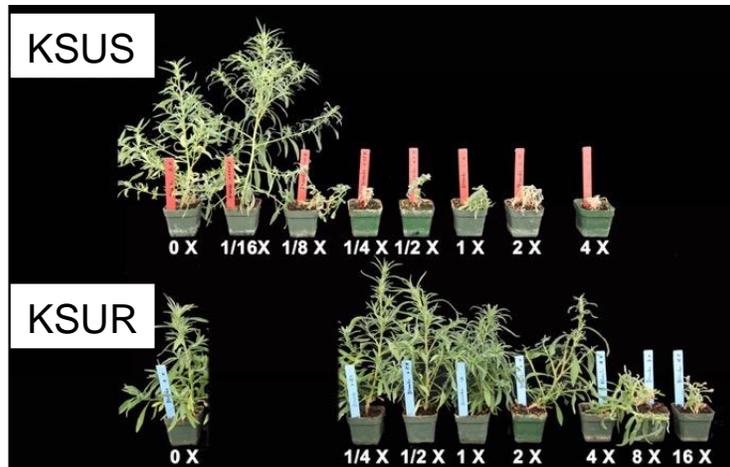


Figure 2.4 Photos of KSUS and KSUR kochia after treated with different doses of dicamba (X=560 g ae ha⁻¹) at 4 WAT.

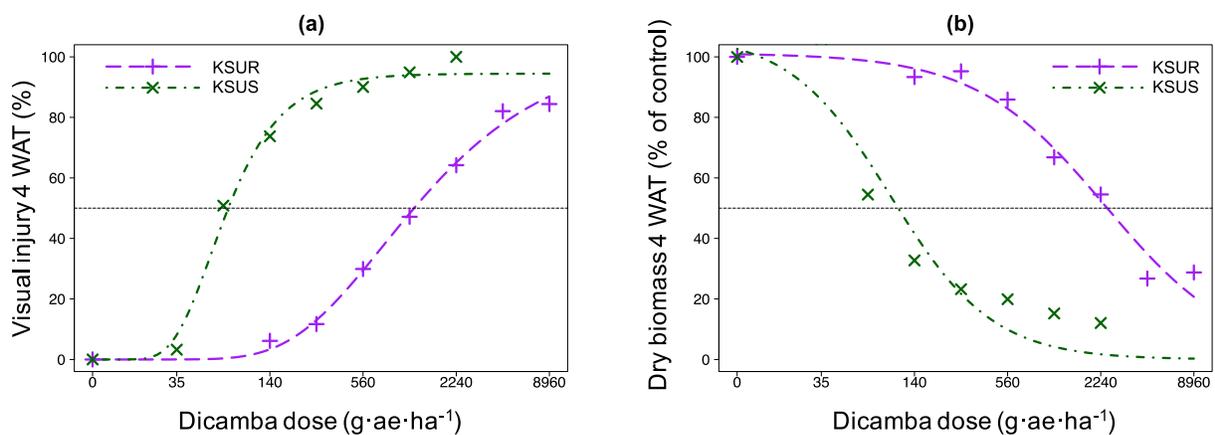


Figure 2.5 Dose-response of KSUS and KSUR kochia to dicamba as measured by (a) visual injury, (b) dry biomass. (Non-linear regression model: $Y=C+(D-C)/(1+\exp[b(\log(x)-\log(I_{50}))])$).

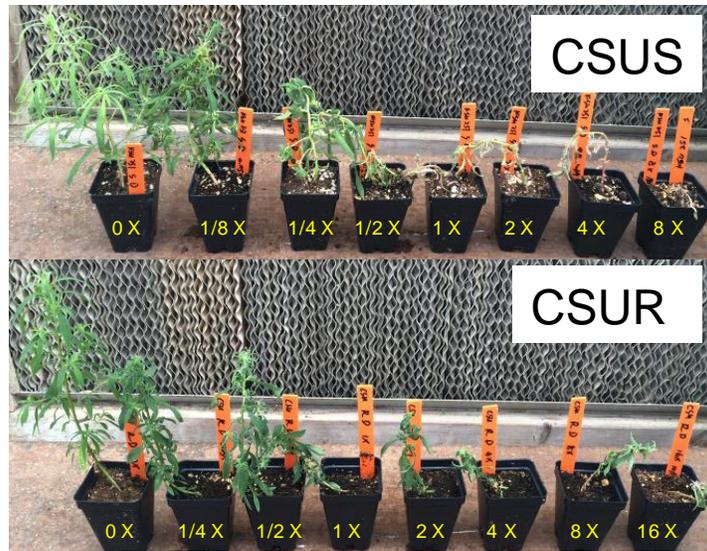


Figure 2.6 Photos of CSUS and CSUR kochia after treated with different doses of dicamba (X=560 g ae ha⁻¹) at 4 WAT.

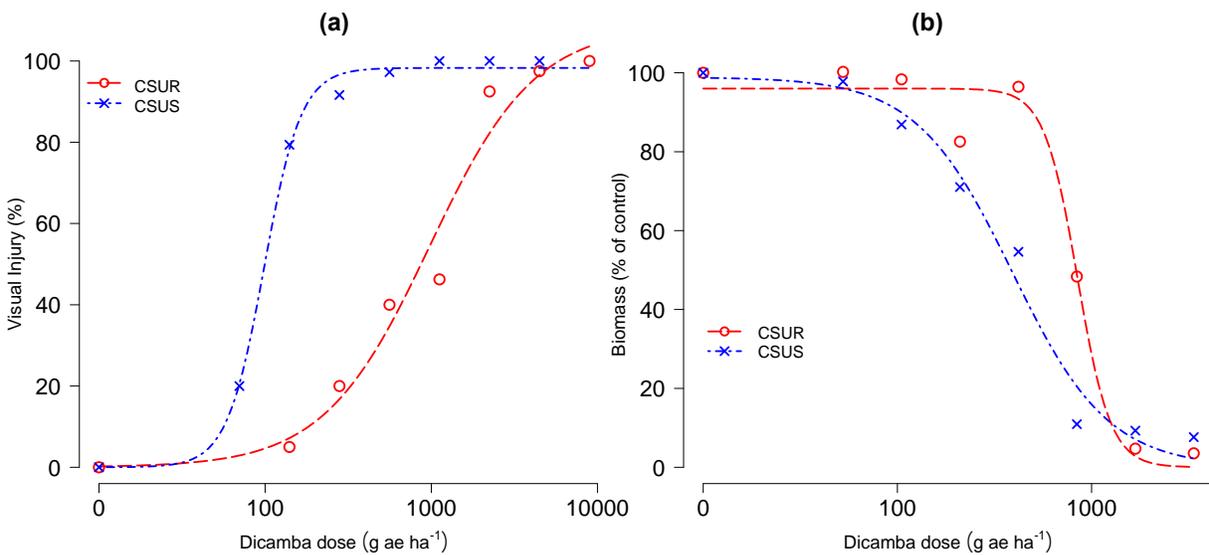


Figure 2.7 Dose-response of CSUS and CSUR kochia to dicamba as measured by (a) visual Injury, (b) dry biomass. (Non-linear regression model: $Y=C+(D-C)/(1+\exp[b(\log(x)-\log(I_{50}))])$).

2.4.2 Absorption, Translocation, and Metabolism of [¹⁴C] Dicamba

The data of [¹⁴C] dicamba absorption and translocation indicate that >90% of dicamba was absorbed by kochia plants within 48 hours after the treatment (HAT, Fig 2.8). Plants of all kochia populations, i.e. KSUR, KSUS, CSUR, and CSUS showed similar amount of dicamba absorption. Therefore, differential absorption of dicamba does not appear to contribute to dicamba resistance in both KSUR and CSUR. However, the translocation pattern of [¹⁴C] dicamba (Fig. 2.9) was found different between these two populations. While the translocation of dicamba in KSUR, KSUS, and CSUS kochia increased from approximately 20% at 24 HAT to 35, 50, and 60%, at 48, 72, and 96 HAT, respectively, the translocation of dicamba in CSUR kochia increased from 8% at 24 HAT to only 10, 14, and 22%, at 48, 72, and 96 HAT, respectively. Even at 336 HAT, there was only 41% of dicamba translocated in the CSUR kochia, which was significantly less than the translocation in CSUS kochia (Fig. 2.9A). Additionally, less dicamba was translocated to above and below the treated leaves in CSUR (Fig. 2.9B and D). For example, 30% less dicamba was translocated to ATL at 48, 72, and 96 HAT in CSUR than CSUS kochia. Significantly more dicamba (30-40%) was retained in the TL of CSUR (Fig. 2.9C), and as a result there was clearly reduced translocation of dicamba into ATL and BTL in this population compared to the other three kochia populations (Fig. 2.9C).

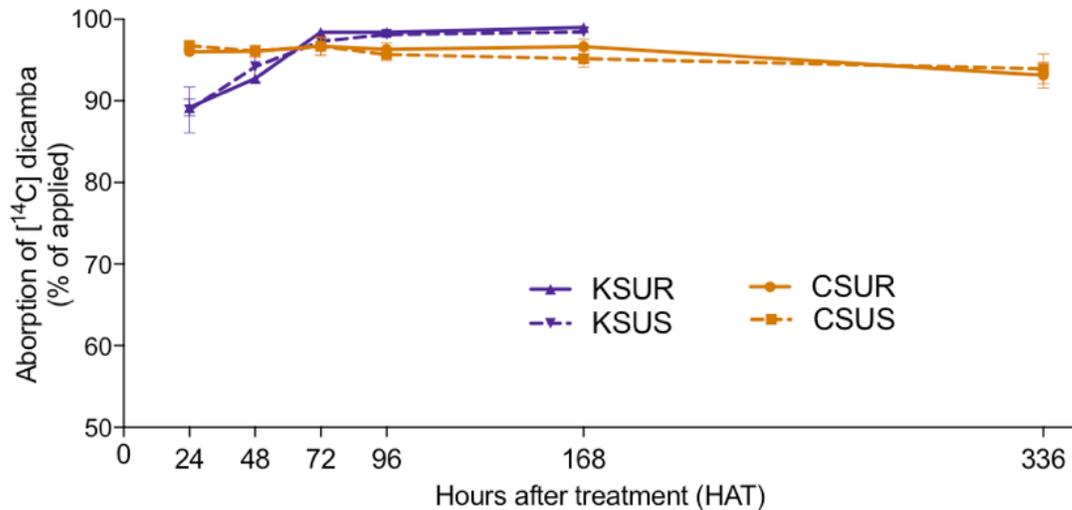


Figure 2.8 Absorption of [^{14}C] dicamba (% of applied) in dicamba-resistant and dicamba-susceptible kochia populations from Kansas and Colorado.

Unlike in CSUR, there was no significant difference in absorption, total translocation, or dicamba distribution in ATL and TL in KSUR compared to susceptible kochia (Fig. 2.8 and 2.9). In contrast to CSUR, an increased translocation of dicamba to BTL was observed in KSUR (Fig. 2.9D). At 72, 96, and 168 HAT, there was about 10% more dicamba translocated to the BTL in KSUR kochia compared to the two dicamba-susceptible kochia populations (Fig. 2.9D). Since our preliminary data showed only <5% of dicamba translocated to the roots, the contribution of increased translocation of dicamba to BTL towards resistance mechanism may be negligible in KSUR kochia.

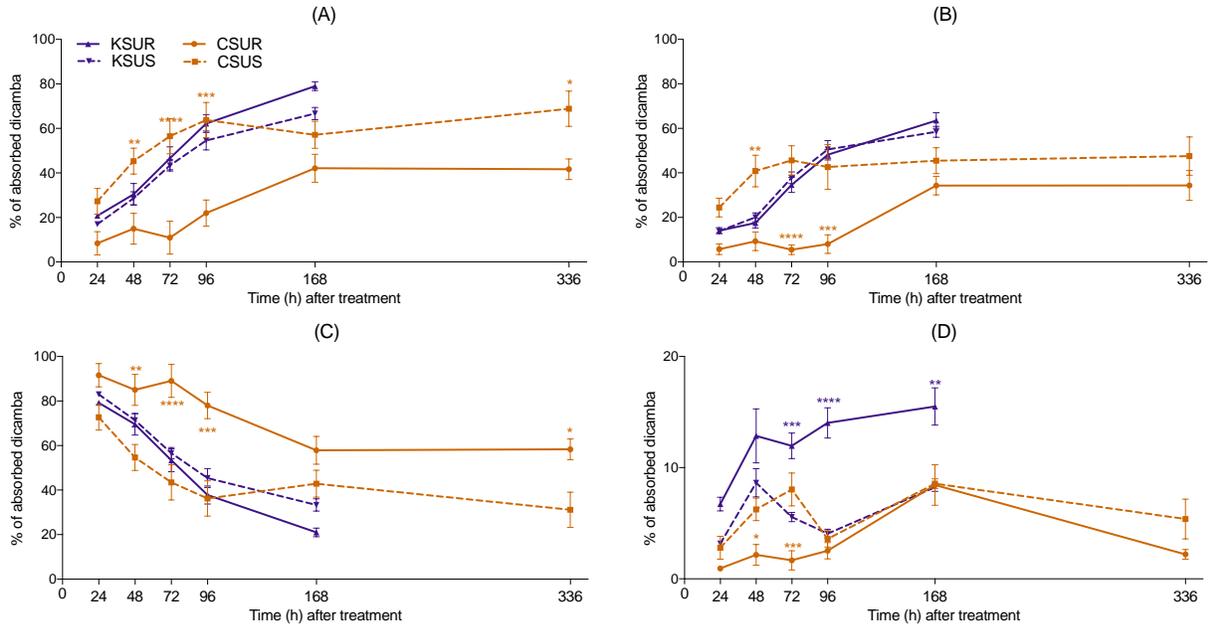


Figure 2.9 Total translocation of $[^{14}\text{C}]$ dicamba (% of absorbed) (A), and the distribution of $[^{14}\text{C}]$ dicamba (% absorbed) to different plant parts, i.e. above treated leaf (B); treated leaf (C); and plant parts below the treated leaf (D) in dicamba-resistant and -susceptible kochia populations from Kansas and Colorado

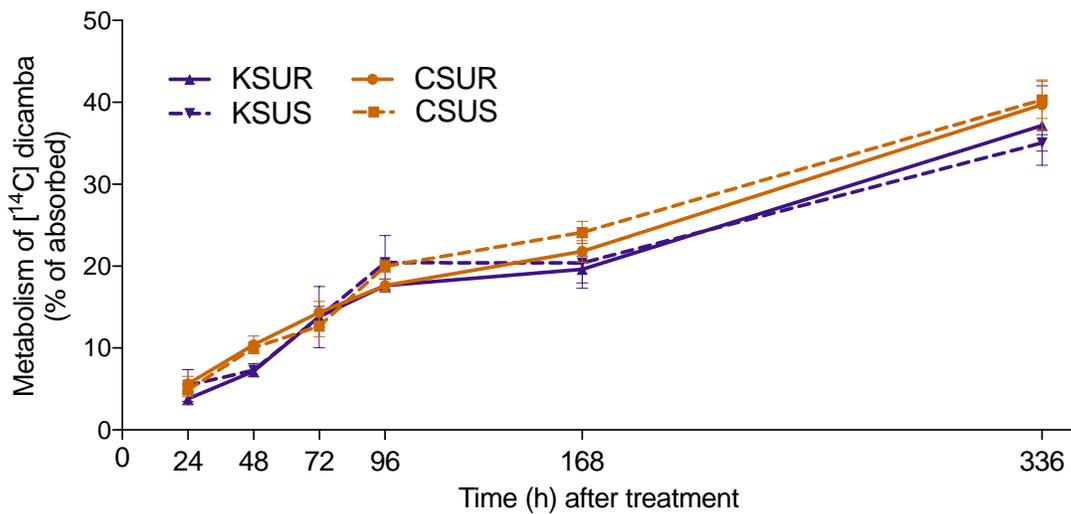


Figure 2.10 Metabolism of $[^{14}\text{C}]$ dicamba in dicamba-resistant and -susceptible kochia populations from Kansas and Colorado.

The [¹⁴C] dicamba metabolism of KSUR, CSUR, KSUS, and CSUS revealed that the degradation of dicamba in all kochia plants increased from approximately 5% at 24 HAT to 10, 15, 20, 25, and 35% at 48, 72, 96, 168, and 336 HAT, respectively (Fig. 2.10). Therefore, there was no difference in the amount of dicamba metabolized among the four kochia populations. Moreover, the pattern of dicamba metabolism as shown in the chromatographs (Fig. 2.11) was also similar in all the four populations. The retention time of the major metabolite of dicamba was at 8.7 min, which is consistent with earlier reports (Broadhurst et al., 1966; Cranston et al., 2001), and this metabolite is expected to be the 5-hydroxy dicamba (2,5-dichloro-3-hydroxy-6-methoxybenzoic acid) (Broadhurst et al., 1966). Therefore, the metabolism of dicamba does not appear to contribute to the resistance in either KSUR or CSUR.

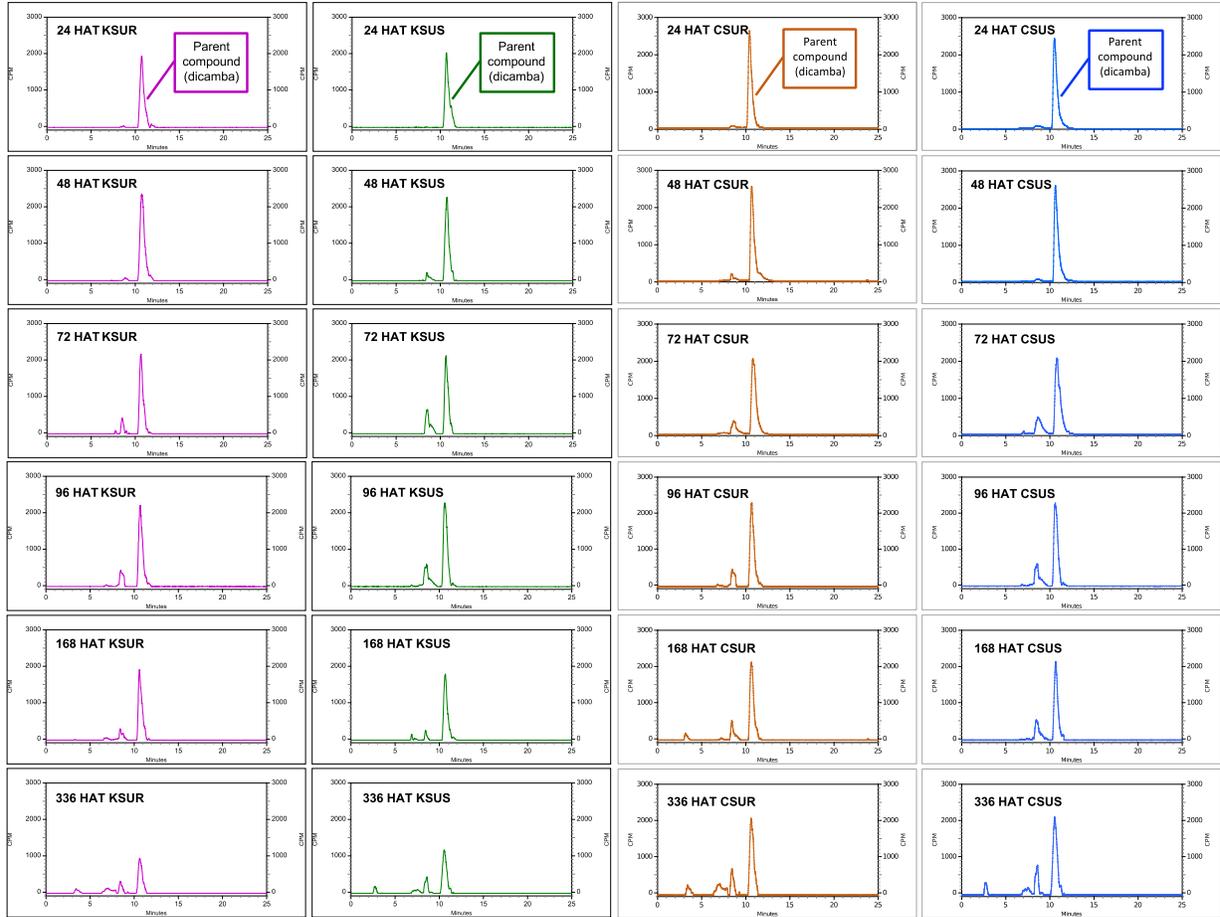


Figure 2.11 Chromatographs of $[^{14}\text{C}]$ dicamba in dicamba-resistant and dicamba susceptible kochia populations from Kansas and Colorado.

2.4.3 Phosphor Image Analysis

Phosphor image analysis also confirmed the same translocation pattern as discussed before in [¹⁴C] dicamba uptake and translocation section. The CSUR kochia had less [¹⁴C] dicamba translocation throughout the plant and most of dicamba was retained on the treated leaf both at 48 and 96 HAT (Fig. 2.12).

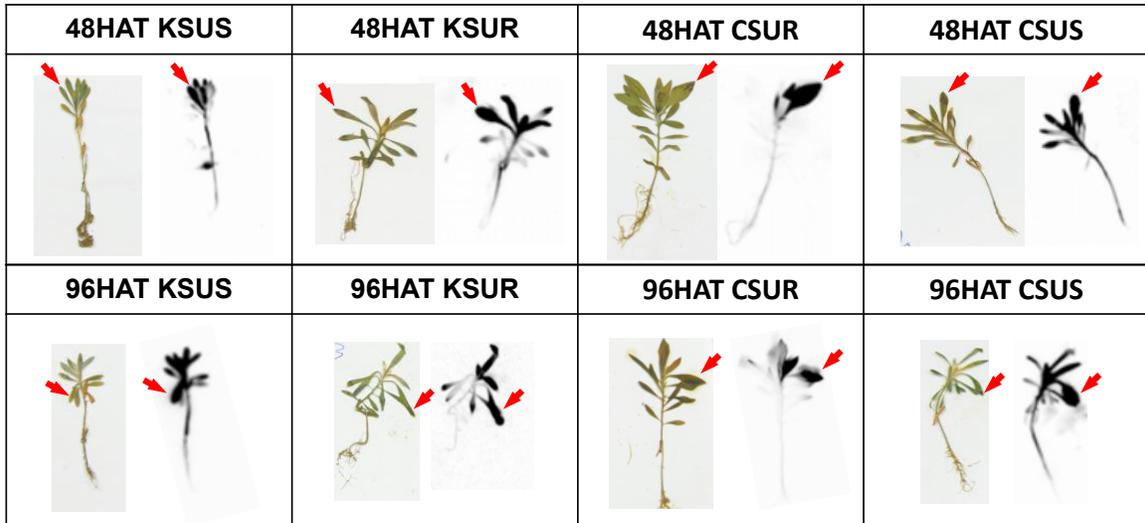


Figure 2.12 Phosphor image analysis of [¹⁴C] dicamba translocation at 48 and 96 hours after treatment (HAT) in KSUS, KSUR, CSUR, and CSUS kochia. Each plant shown in RGB (left) and phosphor image (right). The darker color represents more radioactivity. Arrow points to the leaf where [¹⁴C] dicamba was applied.

2.4.4 TIR1 Homologs Expression and Sequencing

The gene expression analyses of the four TIR1 homologs *QNA3015*, *QNA3953*, *QNA6948*, and *QNA66* (Fig. 2.13) indicate no up- or down-regulation of these genes in all dicamba-resistant and -susceptible kochia populations tested relative to *ALS*, the reference gene used. These results suggest there is no over expression of auxin or dicamba target protein (TIR1 or AFBs) in kochia.

Therefore, it is unlikely that the dicamba resistance in the two populations is endowed by up-regulation of auxin or dicamba target genes.

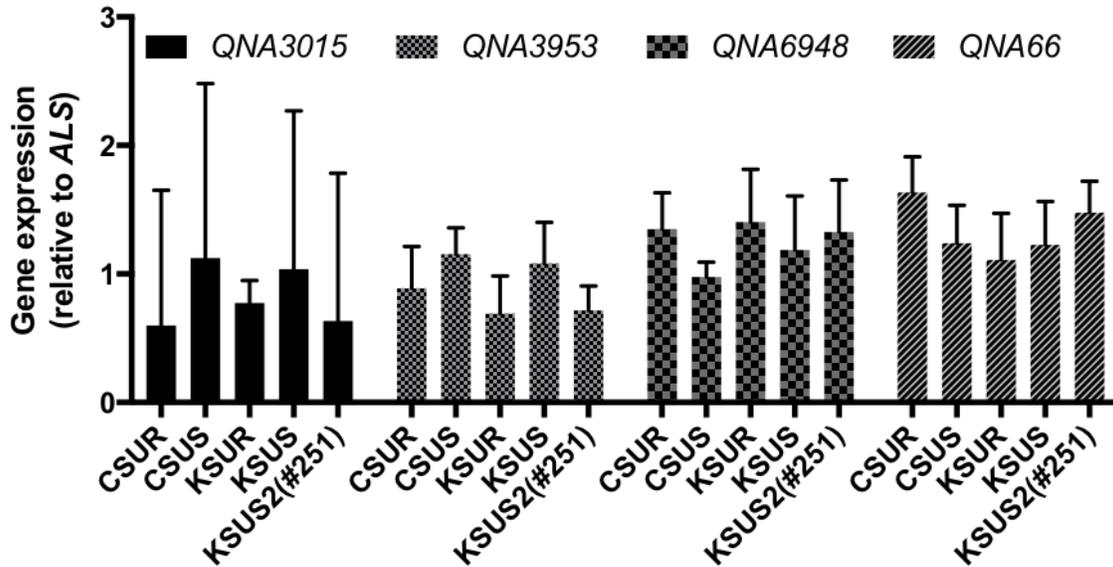


Figure 2.13 Expression profiles of *QNA3015*, *QNA3953*, *QNA6948*, and *QNA66* relative to *ALS* gene in CSUR, CSUS, KSUR, KSUS, and KSUS2(#251) kochia populations (Error bars represent the standard deviation of three technical replicates of three biological replicates)

Sequencing of *QNA3015*, *QNA3953*, *QNA6948*, and *QNA66* genes showed a single nucleotide polymorphism (SNP) between KSUS and KSUR plants only in *QNA6948* (Fig. 2.14), but not in the other genes tested. Although presence of this SNP needs to be tested in a large number of plants as well as a segregating population, these preliminary results suggest a Glu428Asp substitution (as a result of a SNP) in the TIR1 protein in KSUR may contribute to the resistance. Further research is needed to determine the function of this SNP in dicamba resistance in KSUR.

(A)

	2401	Glu ₄₂₈ to Asp ₄₂₈			2450
<i>A. thaliana</i> : gDNA	AGAGTGTTC	CGTCCGAGCC	TTTTGTCATG	GAACCAAATG	TGGCATTGAC
<i>A. thaliana</i> : cDNA	AGAGTGTTC	CGTCCGAGCC	TTTTGTCATG	GAACCAAATG	TGGCATTGAC
<i>B. scoparia</i> : cDNA	CGCGTCTTC	CTTCTGAGCC	TTATGTTCAA	GAACCAAATG	TTTGCTTAAC
KSUR1: cDNA	CGCGTCTTC	CTTCTGATCC	TTATGTTCAA	NAANCAAATG	TTTGCTTAAC
KSUR2: cDNA	CGCGTCTTC	CTTCTGATCC	TTATGTTCAA	GAACCAAATG	TTTGCTTAAC
KSUS3: cDNA	CGCGTCTTC	CTTCTGAGCC	TTATGTTCAA	GAACCAAATG	TTTGCTTAAC
KSUS1: cDNA	CGCGTCTTC	CTTCTGAGCC	TTATGTTCAA	GAACCAAATG	TTTGCTTAAC
KSUS2: cDNA	CGCGTCTTC	CTTCTGAGCC	TTATGTTCAA	GAACCAAATG	TTTGCTTAAC

(B)

	401	Glu ₄₂₈ to Asp ₄₂₈			450
<i>A. thaliana</i> :TIR1_AT3G62980	LDYIEDAGLE	VLASTCKDLR	ELRVFPSEPF	VMEPNVALTE	QGLVSVSMGC
<i>B. scoparia</i> :QNA6948	LDYIEDCGLE	VLAESCKDLR	ELRVFPSEPY	VQEPNVCLTE	QGLVSVAMGC
KSUR1	LDYIEDCGLE	VLAESCKDLR	ELRVFPSEDPY	VQXXNVCLTE	QGLVSVAMGC
KSUR2	LDYIEDCGLE	VLAESCKDLR	ELRVFPSEDPY	VQEPNVCLTE	QGLVSVAMGC
KSUS1	LDYIEDCGLE	VLAESCKDLR	ELRVFPSEPY	VQEPNVCLTE	QGLVSVAMGC
KSUS2	LDYIEDCGLE	VLAESCKDLR	ELRVFPSEPY	VQEPNVCLTE	QGLVSVAMGC
KSUS3	LDYIEDCGLE	VLAESCKDLR	XLRVFPSEPY	VXEPNVCLTE	QGLVSVAMGC

Figure 2.14 Nucleotide (A) and amino acid (B) sequence alignment of *QNA6948* gene fragment of dicamba-resistant (KSUR) and dicamba-susceptible (KSUS) kochia. Nucleotide/amino acid numbering refers to the *Arabidopsis thaliana* TIR1_AT3G62980.1 gene, and *Bassia scoparia* (KSUS kochia) *QNA6948* gene sequence. Nucleotide/amino polymorphism does not exist among *Arabidopsis thaliana*, *Bassia scoparia*, and dicamba susceptible kochia (KSUS) at No. 428 amino acid in the protein sequence.

2.4.5 Protein Studies

To study possible difference in affinity of dicamba at the potential target site conferring resistance, the total protein of KSUR and KSUS kochia was extracted. The SDS-PAGE analysis indicates extraction of good quality of the native proteins from both populations (Fig. 2.15A). Further, the results of Native-PAGE (Fig. 2.15B) demonstrated that the methods of protein extraction and electrophoresis used are compatible with kochia protein analysis. Also, the electrophoretic transfer was successful (data not shown), after the incubation of native protein with [¹⁴C] labelled dicamba. Because of the low sensitivity of phosphor image analysis and potentially the low concentration of dicamba target protein, the method described above did not

provide any conclusive results to suggest if difference in dicamba affinity at potential target proteins contributes to resistance in kochia.

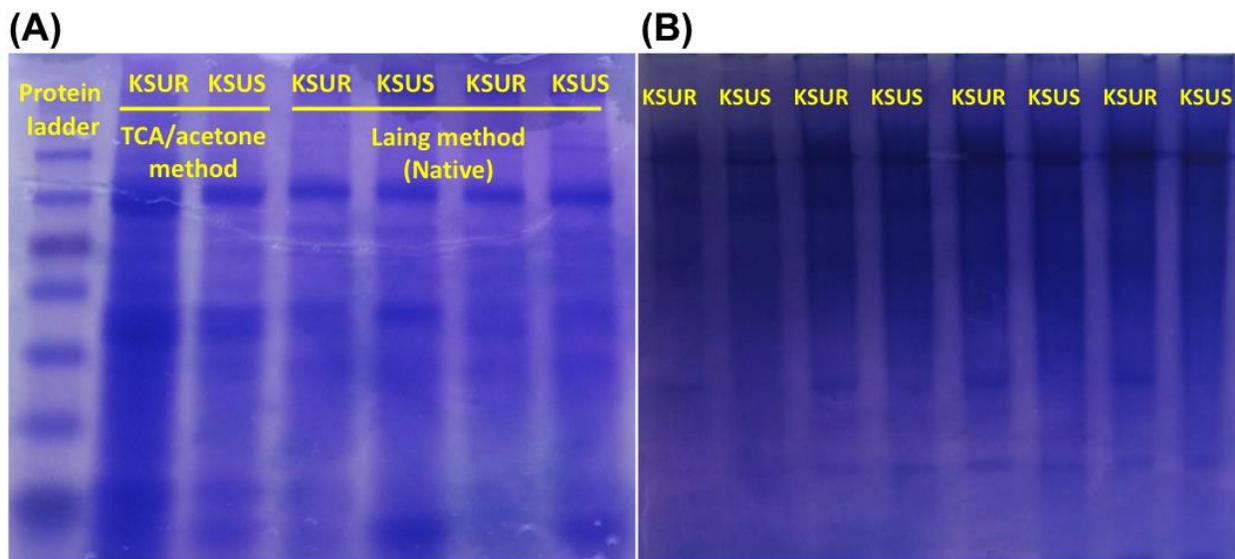


Figure 2.15 SDS-PAGE: quality test of total native protein of kochia compared with denatured protein that was extracted with TCA/acetone method (A), Native-PAGE of native protein extracted from KSUR and KSUS kochia (B).

In summary, the results of this part of the dissertation revealed that the dicamba resistance in CSUR is contributed by limited translocation of dicamba and not in KSUR. Further transcriptome analysis of our collaborative research with Colorado State University (Pettinga et al., 2017) indicated a two-fold upregulation of CHS, an enzyme that regulates synthesis of the flavonols quertecin and kaemperfol that can compete with auxin for intercellular movement and vascular loading via ABCB membrane transporters. In turn this response could be contributing to the reduced dicamba translocation in CSUR kochia due to upregulated flavonols quertecin and kaemperfol synthesis to compete with dicamba for long distance translocation in the dicamba-resistant plants. However reduced translocation of dicamba was not found in KSUR. In contrast, a mutation in the target gene (*TIR1* gene) potentially may confer dicamba resistance in KSUR. If

the future research provide conclusive evidence of co-segregation of this mutation with dicamba resistance phenotype in KSUR, this could be the second case of altered target site, in addition to the first case, where a double mutation in auxin co-receptor *AUX/IAA* gene, that contribute to dicamba resistance (LeClere et al., 2017). Also, if the presence of SNP is confirmed to be associated with dicamba resistance, this will be the first case which substantiates that the auxin receptor TIR1 protein is the target site of dicamba in kochia, and most likely may also be in other species.

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Chapter 3 - Investigation of Genetic Basis of Dicamba Resistance

3.1 Dicamba-Resistant Genes in Kochia from Kansas and Colorado Are Not Linked

3.1.1 Abstract

Kochia is an economically important weed of the US Great Plains. Evolution of resistance to glyphosate in kochia is a major challenge for sustainability of glyphosate-resistant crop technology. Dicamba offers a viable option to manage glyphosate-resistant kochia. However, the recent and rapid evolution of dicamba resistance in glyphosate-resistant kochia populations in KS, CO, and many other states in the US is a serious threat for the management of this weed. The results of chapter 2, clearly suggest that two different mechanisms confer dicamba resistance in KS (KSUR) and CO (CSUR) kochia. While reduced translocation of the dicamba contributes to resistance in CSUR, potentially a SNP in one of *TIRI* homolog may have a significant role in the evolution of dicamba resistance in KSUR kochia. Therefore, the objectives of this research, were to determine a) the inheritance of dicamba resistance in KSUR and b) if the dicamba resistance traits in KSUR and CSUR are linked? The F₁ and F₂ progenies from two different cross combinations, i.e., a) KSUS × KSUR, and b) KSUR × CSUR were generated. Dicamba-dose response and phosphor image analysis were conducted using the F₁ and F₂ progenies. The results of these studies indicate that a) dicamba resistance in KSUR is controlled by an incompletely dominant nuclear gene, and b) the genes conferring dicamba resistance in KSUR and CSUR are not linked. These results provide additional evidence that different populations of kochia can evolve resistance to dicamba by different mechanisms. Therefore, for the efficient

management of this weed, integrated weed management approaches should be adopted to prevent further development of herbicide resistance.

3.1.2 Introduction

Dicamba, a benzoic acid synthetic auxin herbicide is one of the major herbicides used to control kochia after the wide spread of glyphosate resistance in kochia populations in the North American Great Plains. Due to intensive selection, dicamba-resistant (DR) kochia has evolved in this region. After the initial reports of dicamba resistance in MN, ND, ID, and CO, in the 1990s (Heap, 2017), dicamba-resistant kochia has become prevalent in wheat-fallow fields in CO and KS.

Results of physiological and molecular analyses of dicamba resistance in KS (KSUR) and CO (CSUR) kochia (chapter 2) suggest that reduced translocation of dicamba with a double mutation in auxin co-receptor *Aux/IAA* gene (LeClere et al., 2017; Pettinga et al., 2017), and single nucleotide polymorphism (SNP) in auxin receptor *TIR1* gene confer resistance to dicamba in CSUR and KSUR, respectively. Previously, Preston et al. (2009) reported that the dicamba resistance in CSUR kochia is inherited by a single allele with a high degree of dominance. However, the inheritance of dicamba resistance in KSUR is not known. Also, since the results of chapter 2 clearly demonstrate that KSUR and CSUR exhibit two different mechanisms of dicamba resistance, this research was conducted based on the hypothesis that two different genes control these two mechanisms of dicamba resistance in kochia. The objectives of research were a) characterize the inheritance of dicamba resistance in KSUR kochia and b) investigate the interaction of dicamba-resistant genes in KSUR and CSUR kochia.

3.1.3 Materials and Methods

In 2012, kochia seed were collected from a field in Haskell County, Kansas (37°29'48.5"N, 100°46'53.0"W) (Brachtenbach, 2015). Kochia plants generated from these seeds were self-pollinated in isolation, and upon maturity, seed were harvested separately from each of 10 plants. One hundred seedlings were generated separately from seed harvested from each of the above 10 plants. When plants reached 10-12 cm height, 50 plants from each parent were treated with a field rate of dicamba (560 g ae ha⁻¹). In response to dicamba treatment, all the progeny of a single plant that were found susceptible to dicamba were selected as dicamba-susceptible kochia (KSUS). The remaining seed harvested from the KSUS mother plant was used in this research. Likewise, all the progeny of single plant that were resistant to the field rate of dicamba were selected as KSUR. Similarly, the rest of the seed harvested from KSUR mother plant was used in this research. Inbred dicamba-resistant (CSUR, also known as 9425R) and dicamba-susceptible (CSUS, also known as 7710S) kochia lines from CO, derived by single-seed descent for four generations followed by bulk seed production for 13 generations (Howatt et al., 2006; Preston et al., 2009) were also used.

Experiments were conducted in the weed science greenhouse attached to the Department of Agronomy at Kansas State University, Manhattan, KS. The following greenhouse conditions were maintained: 25/20 °C (day/night, d/n) temperatures, 60 ± 10% relative humidity, and 15/9 h d/n photoperiod supplemented with 120 µmol m⁻² s⁻¹ illumination provided with sodium vapor lamps.

3.1.3.1 Perform Crosses of KSUR × KSUS and KSUR × CSUR Kochia

Reciprocal crosses of KSUR and KSUS kochia were performed following the procedure as described by Jugulam et al. (2014). In brief, since kochia bears protogynous flowers with

stigma emerging and being receptive for one week ahead of the emergence of the stamens of the same flower, a few branches were randomly selected prior to stigma emergence. All the leaves and apical meristems were removed, and the inflorescence was covered with Lawson 217 pollination bags (Seedburo Equipment Company, Des Plaines, IL, USA). After stigma emergence, pollen of dehisced anthers from plants selected as male parents were transferred on to the stigmas using a sterilized brush. Subsequently, the branches were covered with the pollination bags again. The same pollination procedure was repeated for five days to ensure successful fertilization of stigma and prevent the contamination from “self-pollination,” since the fertilized kochia flower will be dedicated to producing seeds rather than continue to produce pollen and seed at the same time (Khadka, 2017). During the pollination process, if any new buds were developed from the selected branches, they were removed daily to prevent any possible pollen contamination from the same plant. The branches were covered with pollination bags for about 8 weeks until the seeds were matured. Mature F_1 (KSUR×KSUS) and F_1 (KSUS×KSUR) kochia seed were harvested separately from reciprocal crosses and stored at 4°C for further studies. Seven F_2 families were also generated by self-pollination of randomly selected four each of F_1 (KSUR×KSUS) and three F_1 (KSUS×KSUR) kochia that survived a field rate of dicamba at 560 g ha⁻¹ application.

F_1 (KSUR×CSUR) and F_2 (KSUR×CSUR) were also produced following the same procedure as described above. However, only the direct crosses, i.e., KSUR×CSUR were performed but not reciprocals. Primarily because, a) due to possible the inbreeding depression, the growth of CSUR kochia plants is generally slow but will flower early after planting. The prolific flowering pattern makes it nearly impossible to prevent pollen contamination from the same CSUR plant and b) since both, i.e., KSUR and CSUR phenotypes will be dicamba-

resistant, it will be challenging to separate true F₁ from reciprocal crosses, which can also impact the generation of F₂ progeny as well.

3.1.3.2 F₁ and F₂ Kochia Progeny Response to Dicamba Treatment.

The F₁, F₂ and parental kochia plants were grown in the greenhouse maintained under conditions as described above. When the seedlings reached 10-12 cm height, a set of F₁ (KSUR×KSUS), F₁ (KSUS×KSUR), KSUR, and KSUS were treated with dicamba (Clarity[®], BASF Corp., Florham Park, NJ, USA) at 0, 35, 70, 140, 280, 560, 1120, 2240, and 4480 g ha⁻¹ for the dose-response study. Whereas, the F₂ (KSUR×KSUS), F₂ (KSUS×KSUR), F₁ (KSUR×CSUR), F₂ (KSUR×CSUR) kochia were treated with dicamba (Clarity[®], BASF Corp., Florham Park, NJ, USA) at 560 g ha⁻¹. All the treatments were applied as follows. Herbicides were mixed according to the labels and applied using a bench-type sprayer (Research Track Sprayer, De Vries Manufacturing, Hollandale, MN, USA) equipped with a single moving even flat-fan nozzle tip (8002E TeeJet tip, Spraying Systems Co., Wheaton, IL, USA) delivering 187 L ha⁻¹ at 207 kPa in a single pass at 4.85 km h⁻¹. At four weeks after herbicide treatment (WAT), the dicamba-induced visual injury was rated based on the composite visual estimation of growth inhibition, epinasty (downward curling of plant parts), necrosis, and plant vigor on a scale of 0 (no effect) to 100 (plant death). Plants were clipped off at soil level at 4 WAT, and individual plants were placed in separate paper sacks. Dry biomass of oven dried samples at 60 °C for 72 h was determined. Four replicates were included in each treatment in the dose-response experiments. In the single dicamba dose screening, the plants were rated as alive or dead. All the experiments were repeated.

3.1.3.3 Generation of Clone of F₂ (KSUR×CSUR) Progeny and KSUS Kochia

Thirty-two plants of the F₂ (KSUR×CSUR) progeny and four KSUS were grown in the greenhouse maintained under the same conditions as described above. When seedlings were 30 cm height, few branches were randomly selected, and the leaves and auxiliary buds were removed. The branch was excised from the mother plant and the bottom of the branch was treated with 0.10% indole 3-butyric acid powder (Bontone Rooting Powder, Bonide Products Inc., Oriskany, NY, USA), before placing them in a pot (6.5 × 6.5 × 9 cm) containing field soil (silty loam soil, 1.2% OM, Manhattan, KS, USA) and sand mixture (soil:sand=2:1 w/w), which was steam sterilized at 70 °C for 30 minutes in the Hummert's Media Treatment System (Hummert International, Topeka, KS, USA) and treated with 0.1% of (w/v) of fertilizer solution (Miricle-Gro water soluble all-purpose plant food, N:P:K of 24:8:16, Scotts Miracle-Gro Products Inc. Marysville, OH, USA) prior to use. Plants were covered with a transparent plastic cover (Humidome Clear Plastic Propagation Domes, Hummert International, Topeka, KS, USA) to increase the humidity and hasten root production. After three weeks, the vegetative clones were established, and when the clones reached 10-12 cm height, one set of them (32 clones of the F₂ progeny, 4 clones of KSUS plant) were sprayed with 560 g ha⁻¹ dicamba and visual injury was rated at 4 WAT as described above. Another set of the clones was used for phosphor image analysis.

3.1.3.4 Phosphor Image Analysis

A working stock solution of [¹⁴C] dicamba with 3.3 kBq μL⁻¹ of radioactivity, the equivalent of 560 g ha⁻¹ dicamba in a carrier volume of 187 L was prepared. Clones of F₂ (KSUR×CSUR) kochia progenies were treated with 1 μL of the working solution on a newly expanded leaf. At 96 hours after treatment (HAT), the treated clones were gently uprooted, and the roots were

washed with water carefully to remove soil particles. Subsequently, the whole clone was washed twice with 10 mL of 10% (v/v) aqueous ethanol solution that contains 0.5% of Tween-20, and then pressed using a plant press (Lacey et al., 2001) and dried at 60°C for 72 h. The pressed kochia clones were exposed to BAS-IP MS 2040 E Multipurpose Standard Storage Phosphor Screen (GE Healthcare Life Sciences, Pittsburgh, PA, USA) for 24 h, and the screen was read using Bio-Rad Molecular Imager FX (Bio-Rad Laboratories, Inc. Hercules, CA, USA). The phosphor images were processed using Quantity One software (v4.6.9, Bio-Rad Laboratories, Hercules, CA, USA). The RGB images used for visualization were processed in GNU Image Manipulation Program 2.8.20 (GIMP development team, <https://www.gimp.org>). Four replicates were included in each treatment in a complete randomized design, and the experiment was repeated twice.

3.1.3.5 Experimental Design and Data Analysis.

Split-plot design was used in the dicamba dose-response study. Kochia population and dicamba dose were main- and subplot, respectively. Treatments were arranged in a factorial combination with different population and herbicide doses. No interaction between experimental runs was observed; hence, data from all the experiments were pooled prior to analysis. Then, visual injury and dry biomass data were subjected to non-linear regression analysis using four parameters log-logistic model (Seefeldt et al., 1995) in R (v.3.2.1, R Foundation for Statistical Computing, Vienna, Austria) with the drc package (Ritz and Streibig, 2005).

$$Y=C+(D-C)/(1+\exp[b(\log(x)-\log(I_{50}))]) \quad (3.1)$$

In Eqn 3.1, Y refers to the percentage of untreated, C and D are the lower limit and upper limit of the data, respectively, b is the slope, and I_{50} is the dose required for 50% response, which was

used to estimate GR_{50} (effective dose for 50% biomass reduction) values from the dry biomass data.

Completely randomized design was used in the single dose screening experiments. No interaction between experimental runs was observed, and the data from all the experiments were pooled prior to analysis. Chi-square test fitting to different models was analyzed in R (v.3.2.1, R Foundation for Statistical Computing, Vienna, Austria).

3.1.4 Results and Discussion

Table 3.1 Segregation of resistance or susceptibility to dicamba in F₁ and F₂ progenies of reciprocal crosses between KSUR and KSUS kochia.

Kochia	Total plants	Number of survivors		χ^2 Value [#]	P-value [#]
		Expected*	Observed		
KSUR	32	32	32	-	-
KSUS	32	0	0	-	-
F ₁ (KSUR×KSUS)	122	122	122	-	-
F ₁ (KSUS×KSUR)	115	115	115	-	-
F ₂ (KSUR×KSUS)-1	256	192	183	1.688	0.1939
F ₂ (KSUR×KSUS)-2	128	96	88	2.667	0.1025
F ₂ (KSUR×KSUS)-3	128	96	97	0.042	0.8383
F ₂ (KSUR×KSUS)-4	128	96	91	1.042	0.3074
F ₂ (KSUS×KSUR)-1	256	192	188	0.333	0.5637
F ₂ (KSUS×KSUR)-2	128	96	100	0.667	0.4142
F ₂ (KSUS×KSUR)-3	128	96	93	0.375	0.5403
F ₂ Pooled	1152	840	864	1.838	0.1752

* Expected numbers are based on the allele for dicamba resistance conferring being dominant. #

χ^2 test for goodness of fit to dicamba-resistant : dicamba-susceptible=3:1.

All plants of F₁ progeny from reciprocal crosses F₁ (KSUR×KSUS) and F₁ (KSUS×KSUR) survived field dose of dicamba (560 g ha⁻¹) treatment (Table 3.1), suggesting that the dicamba resistance is nuclear inherited and can be transmitted both via maternal and paternal parents. The genetic analysis of F₂ (KSUR×KSUS) and F₂ (KSUS×KSUR) families, fits the Mendelian segregation of single dominant nuclear gene (3:1 for dicamba-resistant and -susceptibility) with the *P-values* of χ^2 test larger than 0.1 (Table 3.1). Furthermore, the results of the dose-response study indicate that the level of dicamba resistance of F₁ progeny derived from reciprocal crosses, i.e., F₁ (KSUR×KSUS) and F₁ (KSUS×KSUR) was close to the KSUR parent (Fig. 3.1). However, the resistance indices (RI) of dicamba relative to KSUS parent (Table 3.2) were 8.1, 6.9, and 5.9 for KSUR, F₁ (KSUR×KSUS), and F₁ (KSUS×KSUR) kochia, respectively, and the RI for F₁ (KSUR×KSUS), and F₁ (KSUS×KSUR) were significantly lower than KSUR kochia. These data suggest that the level of dicamba resistance was decreased when KSUR kochia was crossed with KSUS kochia, and hence the dicamba resistance is not a completely dominant trait. These results concur with previous report of inheritance of dicamba resistance in CSUR (Preston et al. 2009), suggesting that an incomplete dominant nuclear gene controls dicamba resistance.

Table 3.2 Estimated values of GR₅₀ (effective dose for 50% biomass reduction) and RI (resistant index) for F₁ (KSUR×KSUS) and F₁ (KSUS×KSUR) kochia progeny, and KSUR relative to the KSUS kochia*.

Kochia	GR ₅₀ (g ha ⁻¹) ⁺	Resistant index (RI) [#]
KSUS	180(10)	-
KSUR	1456(57)	8.1 a
F ₁ (KSUR×KSUS)	1247(67)	6.9 b
F ₁ (KSUS×KSUR)	1066(40)	5.9 c

* The four parameters log-logistic model was used for estimation: $Y=C+(D-C)/(1+\exp[b(\log(x)-\log(I_{50}))])$; ⁺ Values are presented in mean value (standard error); [#] values followed by different letters are significantly (P<0.05) different in the column.

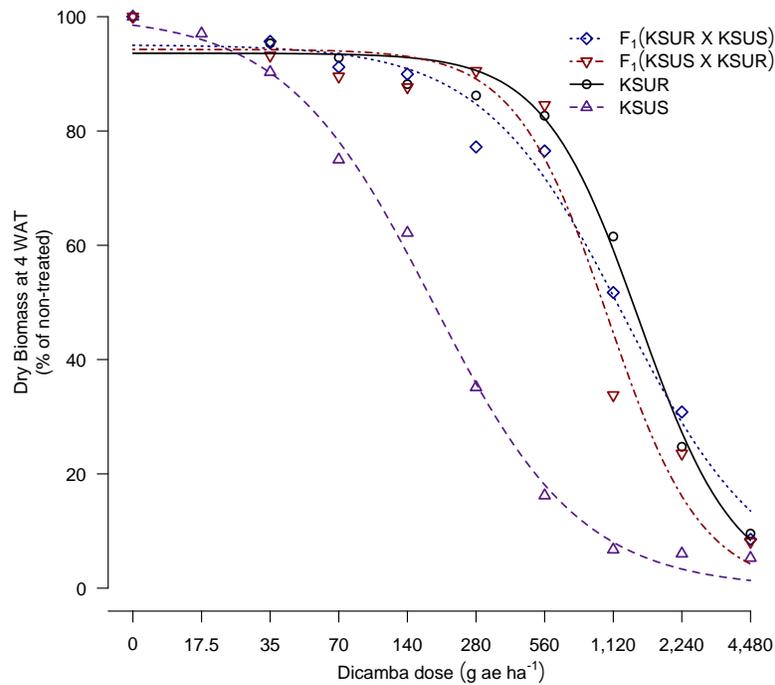


Figure 3.1 Whole plant dicamba dose-response of F₁ progeny derived from reciprocal crosses, i.e. F₁ (KSUR×KSUS) and F₁ (KSUS×KSUR) and, KSUR and KSUS kochia.

As described earlier, two different incompletely dominant nuclear genes confer the dicamba resistance in KSUR and CSUR kochia and therefore, as expected the F₁ (KSUR×CSUR) progeny were all found resistant to dicamba (Table 3.3). Furthermore, the segregation of the phenotypes as dicamba-resistant or-susceptible in F₂ (KSUR×CSUR) progeny followed the inheritance pattern of two unlinked dominant nuclear genes (Fig. 3.2). The χ^2 test for goodness of fit to resistant: susceptible (15:1) (Table 3.3) had a *P-value* equals to 0.1232 (>0.10), which provides additional evidence that two different genes control dicamba resistance in KSUR and CSUR and hence the presence of two distinct mechanisms of dicamba resistance in these kochia populations (chapter 2).

Table 3.3 Segregation of resistance or susceptibility to dicamba (560 g ha⁻¹) in F₁ (KSUR×CSUR), F₂ (KSUR×CSUR), KSUR, KSUS, CSUR, and CSUS kochia.

Kochia	Total plants	Number of survivors		χ^2 Value [#]	<i>P-value</i> [#]
		Expected*	Observed		
KSUR	32	32	32	-	-
KSUS	32	0	0	-	-
CSUR	32	32	32		
CSUS	32	0	0		
F ₁ (KSUR×CSUR)	66	66	66	-	-
F ₂ (KSUR×CSUR)	352	330	323	2.376	0.1232

* Expected numbers are based on the allele for resistance to dicamba being dominant in both populations and that the two genes are not linked. [#] χ^2 test for goodness of fit to dicamba-resistant:dicamba-susceptible (15:1).

F ₂ genotype	KC	Kc	kC	kc
KC	KKCC	KKCc	KkCC	KkCc
Kc	KKCc	KKcc	KkCc	Kkcc
kC	KkCC	KkCc	kkCc	kkCc
kc	KkCc	Kkcc	kkCc	kkcc

(K-Kansas resistant gene, C-Colorado resistant gene)

Phenotype:  Resistant  Susceptible

Figure 3.2 Illustration of dicamba-resistant and -susceptible genotypes and phenotypes as well as the pattern of Mendelian inheritance of two unlinked dominant nuclear genes in F₂ (KSUR×CSUR) progeny, in response to the label recommended field use rate of dicamba (560 g ha⁻¹).

The results of chapter 2 also indicate the reduced translocation of dicamba conferring dicamba resistance only in CSUR but not KSUR. Therefore, in response to 560 g ha⁻¹ of dicamba treatment, the clones of F₂ (KSUR×CSUR) progeny are expected to exhibit the following three phenotypes: a) dicamba-resistant and reduced translocation (plants possessing the CSUR gene regardless of the presence of KSUR gene), b) dicamba-resistant and normal translocation (plants do not possess the CSUR but only the KSUR gene), and c) dicamba-susceptible and normal translocation (plants with no resistance gene) (Fig. 3.3).

F ₂ genotype	KC	Kc	kC	kc
KC	KKCC	KKCc	KkCC	KkCc
Kc	KKCc	KKcc	KkCc	Kkcc
kC	KkCC	KkCc	kkCc	kkCc
kc	KkCc	Kkcc	kkCc	kkcc

(K-Kansas resistant gene, C-Colorado resistant gene)

Phenotypes:  Dicamba-resistant, Reduced translocation
 Dicamba-susceptible, Normal translocation
 Dicamba-resistant, Normal translocation

Figure 3.3 Illustration of dicamba-resistant and -susceptible phenotypes and the pattern of inheritance of two unlinked dominant nuclear genes in F₂ (KSUR×CSUR) progeny using single-dose (dicamba at 560 g ha⁻¹) selection and phosphor image analysis with [¹⁴C] labeled dicamba.

As expected, the analyses of the data (Fig. 3.4) and phosphor imaging with [¹⁴C] labelled dicamba (Fig. 3.4) in comparison with KSUS kochia (known to have dicamba-susceptible and normal translocation phenotype; Fig. 3.4A), identified all the above three phenotypes in response to 560 g ha⁻¹ of dicamba treatment. Although, only nine plants of F₂ progeny were tested in phosphor image analysis, to test if the ratios of the three phenotypes, i.e. dicamba-resistant and reduced translocation : dicamba-resistant and normal translocation : dicamba-susceptible and normal translocation, however the results fit the Mendelian segregation of 12:3:1. A more extensive phosphor image analysis on F₂ plants is needed.

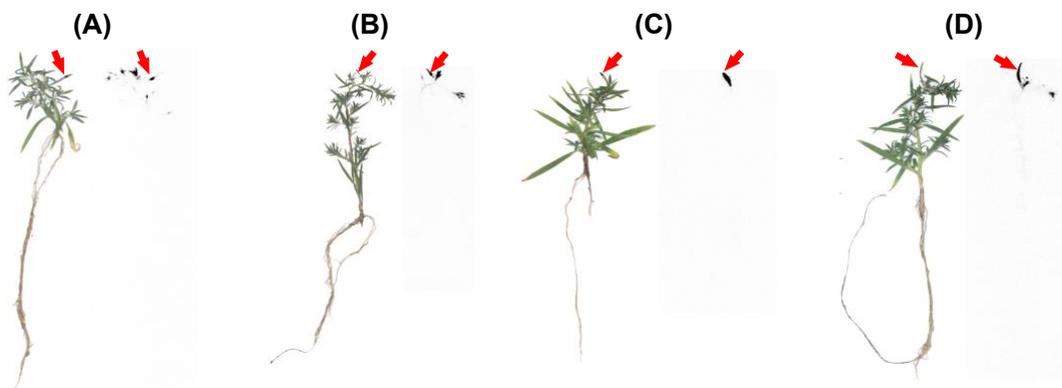


Figure 3.4 Phosphor images of [^{14}C] dicamba in clones of F_2 (KSUR \times CSUR) progeny and KSUS at 72 hours after treatment (HAT). (A) KSUS, dicamba-susceptible and normal translocation; (B) F_2 (KSUR \times CSUR), dicamba-susceptible and normal translocation; (C) F_2 (KSUR \times CSUR), dicamba-resistant and reduced translocation; (D) dicamba-resistant and normal translocation. Each plant is shown in RGB image (left) and phosphor image (right). In phosphor image, the darker color represents more radioactivity. Arrow points at the treated leaf on each plant where radioactive herbicide was applied.

In conclusion, the F_1 and F_2 analyses of the reciprocal crosses of KSUR and KSUS kochia demonstrated that the dicamba resistance in kochia from KS is inherited by an incomplete single dominant nuclear gene. This is the first report of the inheritance of dicamba resistance in kochia from KS. Furthermore, in response to dicamba (560 g ha^{-1}) the F_1 and F_2 progeny of a cross between KSUR and CSUR kochia, confirmed that the resistance to dicamba in these two populations is controlled by two different genes which are not linked. Overall these results suggest that kochia populations can evolve resistance to dicamba via several mechanisms (non-target-site- and/or target-site based), potentially as a result of different types of dicamba selection pressure. Consequently, it is essential to use integrated weed management methods to control

kochia to minimize further development and spread of herbicide resistance in this economically important weed.

3.2 Glyphosate- and Dicamba-Resistant Genes Are Not Linked in Kochia

3.2.1 Abstract

Kochia is one of the most troublesome weeds in the Great Plains of North America. Its infestation in productive lands can lead to significant yield loss and adversely affect the quality of agricultural crops. Glyphosate and dicamba have been effective options to control kochia for decades. Due to extensive use of these herbicides, many kochia populations across the Great Plains have evolved resistance to glyphosate and/or dicamba. Especially, dicamba-resistant kochia populations are often also found to be glyphosate-resistant in KS. The overall objective of this study was to determine if the resistance to these two herbicides is inherited together as a result of close linkage of these genes. Reciprocal crosses were performed between glyphosate- and dicamba-resistant (GDR) and glyphosate- and dicamba-susceptible (GDS) kochia to produce F₁ and F₂ progeny. Two F₁ and eight F₂ progenies were screened with the label recommended rates of dicamba (560 g ha⁻¹) or glyphosate (840 g ha⁻¹), and additionally, two F₂ progenies were screened with glyphosate followed by dicamba at these field rates sequentially. The results suggest that all F₁ progeny survived both dicamba and glyphosate treatments. Chi-square analyses of F₂ families suggest that a) glyphosate and dicamba resistance in kochia are inherited by a single complete and incomplete dominant nuclear gene, respectively; and b) glyphosate- and dicamba-resistant genes are not linked in kochia. Thus, it appears that the dicamba and glyphosate resistance were evolved separately due to the intense selection of these herbicides. The single dominant nuclear-controlled gene inheritance of dicamba and glyphosate resistance in kochia can spread rapidly both via seed and pollen. Therefore, it is essential to choose effective management tools for kochia control and prevent further selection of higher levels of dicamba and/or glyphosate resistance.

3.2.2 Introduction

Glyphosate is the most widely used herbicide globally, which was first commercialized in 1974 (Franz et al., 1997). Due to its non-selective nature, glyphosate application was not widely adopted in agriculture, until the commercialization of Roundup Ready® technology in crops in the late 1990s (Duke and Powles, 2008; Franz et al., 1997). However, extensive and intensive application of glyphosate in the last two decades has resulted in the evolution of resistance in many weed species (Heap, 2017). Kochia, one of the most economically important weeds of the Central Great Plains in North America, evolved glyphosate resistance following the resistance to PSII-, ALS-inhibitors (Heap, 2017), and synthetic auxin herbicides (Heap, 2017). The first case of glyphosate-resistant (GR) kochia was reported in southwestern KS (Heap, 2017). In last 10 years, 16 populations of GR kochia across the Great Plains of North America have been documented (Heap, 2017). A recent survey of crop advisors in western KS reported the presence of GR kochia in half of the surveyed fields with >50% of frequency, and almost all fields at some level (Godar, 2014). Dicamba has become one of the major herbicides used to control kochia after the rapid spread of GR kochia in the North American Great Plains. However, because of intensive use, dicamba-resistant (DR) kochia has also evolved and spread in this region. Following the evolution of the first cases of dicamba resistance in MT, ND, ID, and CO, in early 1990s (Heap, 2017), the incidence of DR kochia is more common, especially in wheat-fallow fields in CO and KS. Interestingly, most of the DR kochia populations are also found to resistant to glyphosate (Brachtenbach, 2015).

To mitigate the problem of glyphosate- and dicamba-resistant (GDR) kochia in Central Plains agriculture, it is essential to understand the evolution and genetic basis of glyphosate and

dicamba resistance, to help develop management strategies to prevent further selection of higher levels of resistance to these herbicides in kochia and potentially to other herbicides.

Glyphosate resistance in kochia from KS has been shown to be inherited by a single dominant gene (Jugulam et al., 2014). Also, Preston et al. (Preston et al., 2009) reported that the dicamba resistance in kochia from CO is inherited by a single allele with a high degree of dominance. However, Cranston et al. (2001) speculated that the dicamba resistance in kochia from MT is a quantitative trait, resulting from a number of relatively small changes in gene products, such as herbicide binding proteins, transporters, and metabolic enzymes. Nonetheless, the inheritance of dicamba resistance in kochia from KS is unknown, and also the information on behavior of glyphosate-resistant (GR) and dicamba-resistant (DR) genes in the same kochia population is lacking. The GDR kochia from KS can provide a unique opportunity to uncover the linkage of glyphosate and dicamba-resistant genes. Therefore, the focus of this research was to uncover the linkage of glyphosate- and dicamba-resistant genes in GDR kochia (i.e., KSUR of Chapter two). The objectives of this investigation were: 1) determine the inheritance of glyphosate and dicamba resistance in GDR kochia, 2) examine the linkage of glyphosate- and dicamba-resistant genes in GDR kochia.

3.2.3 Materials and Methods

In 2012, kochia seed were collected from a field in Haskell County, Kansas (37°29'48.5"N, 100°46'53.0"W) (Brachtenbach, 2015). Kochia plants generated from these seed were self-pollinated by keeping the plants in isolation from other kochia plants, and upon maturity, seed were harvested separately from each of the 10 plants. One hundred seedlings were generated separately from seed harvested from each of the above 10 plants. When plants reached 10-12 cm height, 50 plants from each parent were treated with a field rate of glyphosate (840 g ha⁻¹) or dicamba (560 g ha⁻¹). In response to glyphosate and dicamba treatment, all the progeny from a single plant that were found susceptible to both glyphosate and dicamba were selected as glyphosate- and dicamba-susceptible (GDS) kochia. The remaining seed harvested from the GDS mother plant was used in all experiments in this research. Likewise, all the progeny from a single plant that were resistant to a field rate of both glyphosate and dicamba were selected as glyphosate- and dicamba-resistant (GDR) kochia. Also, the rest of the seed harvested from GDR mother plant was used in all of the experiments.

Experiments were conducted in the weed science greenhouse attached to the Department of Agronomy at Kansas State University, Manhattan, KS. The following greenhouse conditions were maintained: 25/20 °C (day/night, d/n) temperatures, 60 ± 10% relative humidity, and 15/9 h d/n photoperiod supplemented with 120 μmol m⁻² s⁻¹ illuminations provided with sodium vapor lamps.

3.2.3.1 Reciprocal Crosses of GDR and GDS Kochia

Reciprocal crosses of GDR and GDS kochia plants were performed following the method described previously (Jugulam et al., 2014). In brief, kochia bears protogynous flowers with stigma emergence and receptivity occurring one week ahead of the emergence of the stamens of

the same flower. Therefore, a few branches were randomly selected prior to stigma emergence. After removing all the leaves and apical meristems, the branches were covered with Lawson 217 pollination bags (Seedburo Equipment Company, Des Plaines, IL, USA). After stigma emergence, pollen of dehisced anthers from the selected paternal plant was transferred on to the stigmas using a sterilized brush. Immediately after pollination, the branches were covered with the pollination bags again. The pollination procedure was repeated five times to ensure successful fertilization for stigma, and prevent the contamination from “self-pollination,” since the fertilized kochia flower will be dedicated to producing seeds rather than continue to produce pollen and seed at the same time (Khadka, 2017). Throughout the duration of pollination process, any new buds developed on the selected branches were removed daily to prevent any possible pollen contamination from the same plant. The branches were covered with pollination bags for about 8 weeks until the seed were matured. The mature seed of F₁ (GDR×GDS) and F₁ (GDS×GDR) kochia were harvested separately from reciprocal crosses and stored at 4°C for further studies. Eight F₂ progeny families were generated by self-pollination of randomly selected four F₁ (GDR×GDS) and three F₁ (GDS×GDR) kochia that survived a field rate of glyphosate (840 g ha⁻¹) and followed by dicamba (560 g ha⁻¹) treatment.

3.2.3.2 F₁ and F₂ Kochia Progeny Test with Glyphosate and Dicamba

The F₁ progeny of (GDR×GDS) and (GDS×GDR), as well as GDR and GDS parental kochia were grown in the greenhouse maintained under the same conditions as described above. When the seedlings were 10-12 cm height, they were treated with several doses of dicamba (Clarity[®], BASF Corp., Florham Park, NJ, USA) without AMS at 0, 70, 140, 280, 560, 1120, 2240, 4480, and 8960 g ha⁻¹. Additionally, 50 other plants of F₁ (GDR×GDS) and F₁ (GDS×GDR) kochia were also treated with 840 g ha⁻¹ of glyphosate and 560 g ha⁻¹ of dicamba

separately. However, the F₂ progeny of kochia (Table 3.4) were exposed to three different herbicide treatments: a) 840 g ha⁻¹ of glyphosate with 2.5% (w/v) AMS, b) 560 g ha⁻¹ of dicamba, and c) 840 g ha⁻¹ of glyphosate followed by 560 g ha⁻¹ of dicamba with 2.5% (w/v) AMS.

All the herbicide treatments were applied as follows. Herbicides were mixed according to the label and applied using a bench-type sprayer (Research Track Sprayer, De Vries Manufacturing, Hollandale, MN, USA) equipped with a single moving even flat-fan nozzle tip (8002E TeeJet tip, Spraying Systems Co., Wheaton, IL, USA) delivering 187 L ha⁻¹ at 207 kPa in a single pass at 4.85 km h⁻¹. At four weeks after herbicide treatment (WAT), the dicamba-induced visual injury was rated based on the composite visual estimation of growth inhibition, epinasty (downward curling of plant parts), necrosis, and plant vigor on a scale of 0 (no effect) to 100 (plant death). Whereas, the glyphosate injury was rated based on plant stunting, chlorosis followed by necrosis. Plant were clipped off at soil level at 4 WAT and individual plants were placed in separate paper sacks. Dry biomass was recorded after oven drying the samples at 60 °C for 72 h. Four replicates were included in each treatment and all experiments were repeated.

3.2.3.3 Experimental Design and Data Analysis

Split-plot design was used in dicamba dose-response study. Kochia population and herbicide dose were used as main- and subplot, respectively. Treatments were arranged in a factorial combination with different population and herbicide doses. No interaction between experimental runs was observed; hence, data from the repeated experiments were pooled prior to analysis. Then, visual injury and dry biomass data were subjected to non-linear regression analysis using four parameters log-logistic model (Seefeldt et al., 1995) in R (v.3.2.1, R Foundation for Statistical Computing, Vienna, Austria) with the *drc* package (Ritz and Streibig, 2005).

$$Y=C+(D-C)/(1+\exp[b(\log(x)-\log(I_{50}))]) \quad (3.2)$$

In Eqn 3.2, Y refers to the percentage of untreated, C and D are the lower limit and upper limit of the data, respectively, b is the slope, and I_{50} is the dose required for 50% response, which was used to estimate GR_{50} (effective dose for 50% biomass reduction) values from the dry biomass data.

The completely randomized design was used in the single dose screening experiments. No interaction between experimental runs was observed, and data from the repeated experiments were pooled prior to analysis. Chi-square test of fitting to different Mendelian segregation models were conducted in R (v.3.2.1, R Foundation for Statistical Computing, Vienna, Austria).

3.2.4 Results and Discussion

All F₁ progeny (GDR×GDS or GDS×GDR) of kochia survived glyphosate application at 840 g ha⁻¹ (Table 3.4). Jugulam et al. (2014) reported glyphosate resistance in kochia from KS was inherited by a single dominant nuclear allele. Therefore, no further dose-response of glyphosate on F₁ progeny was conducted. The analysis of segregation of resistance or susceptibility to glyphosate in F₂ (GDR×GDS) and F₂ (GDS×GDR) progeny (Table 3.4) indicate that the glyphosate resistance in this kochia population is also inherited by a single dominant nuclear gene as reported previously (Jugulam et al., 2014), because the *P-value* of the χ^2 test in four each of F₂ (GDR×GDS) and (GDS×GDR) progenies were all greater than 0.1.

Table 3.4 Segregation of resistance to glyphosate and/or dicamba in F₁ and F₂ progenies.

Kochia	Total plants	Number of survivors		χ^2 Value	P-value
		Expected	Observed		
Glyphosate application at 840 g ae ha ⁻¹ ; the χ^2 test for goodness of fit to R:S=3:1					
GDR	32	32	32	-	-
GDS	32	0	0	-	-
F ₁ (GDR×GDS)	32	32	32	-	-
F ₁ (GDS×GDR)	32	32	32	-	-
F ₂ (GDR×GDS)-1	64	48	46	0.333	0.5637
F ₂ (GDR×GDS)-2	64	48	51	0.750	0.3865
F ₂ (GDR×GDS)-3	64	48	49	0.083	0.7728
F ₂ (GDR×GDS)-4	64	48	45	0.750	0.3865
F ₂ (GDS×GDR)-1	64	48	47	0.083	0.7728
F ₂ (GDS×GDR)-2	64	48	43	2.083	0.1489
F ₂ (GDS×GDR)-3	64	48	44	1.333	0.2482
F ₂ Pooled	448	336	325	1.440	0.2301
Dicamba application at 560 g ae ha ⁻¹ ; the χ^2 test for goodness of fit to R:S=3:1					
GDR	32	32	32	-	-
GDS	32	0	0	-	-
F ₁ (GDR×GDS)	122	122	122	-	-
F ₁ (GDS×GDR)	115	115	115	-	-
F ₂ (GDR×GDS)-1	256	192	183	1.688	0.1939
F ₂ (GDR×GDS)-2	128	96	88	2.667	0.1025
F ₂ (GDR×GDS)-3	128	96	97	0.042	0.8383
F ₂ (GDR×GDS)-4	128	96	91	1.042	0.3074
F ₂ (GDS×GDR)-1	256	192	188	0.333	0.5637
F ₂ (GDS×GDR)-2	128	96	100	0.667	0.4142
F ₂ (GDS×GDR)-3	128	96	93	0.375	0.5403
F ₂ Pooled	1152	840	864	1.838	0.1752
840 g ha ⁻¹ of glyphosate followed by 560 g ha ⁻¹ of dicamba; the χ^2 test for goodness of fit to R:S=9:7					
F ₂ (GDR×GDS)-1	256	144	132	2.286	0.1306
F ₂ (GDS×GDR)-1	256	144	150	0.571	0.4497
F ₂ Pooled	512	288	282	0.286	0.5930

All F₁ (GDR×GDS) and F₁ (GDS×GDR) kochia progeny also survived the field dose of dicamba (560 g ha⁻¹) application (Table 3.4), suggesting that the dicamba resistance is a nuclear inherited dominant trait. The genetic analyses of F₂ (GDR×GDS) and F₂ (GDS×GDR) families suggested that the *P-values* of the χ^2 test are >0.1 when the goodness of fit test for 3:1 resistant to susceptible was conducted. These data support the segregation of a single dominant nuclear gene. Furthermore, the analyses of dicamba dose-response results of F₁ kochia (GDR×GDS and GDS×GDR) compared to GDR and GDS parental populations indicate that the level of dicamba resistance in F₁ progeny is close to the GDR kochia (Fig. 3.5). Similar to the results as discussed in section 3.1, the RI values indicate the level of dicamba resistance decreases in the progeny generated from a cross between the GDR and GDS kochia. Therefore, the dicamba resistance in the GDR kochia is an incompletely dominant trait.

Table 3.5 Estimated values of GR₅₀ (effective dose for 50% biomass reduction) and RI (resistant index) for F₁ (GDR×GDS) and F₁ (GDS×GDR) kochia progenies, GDR and GDS kochia populations*.

Kochia	GR ₅₀ (g ae ha ⁻¹) ⁺	Resistant index (RI) [#]
GDS	180(10)	-
GDR	1456(57)	8.1 a
F ₁ (GDR×GDS)	1247(67)	6.9 b
F ₁ (GDS×GDR)	1066(40)	5.9 c

* The four parameters log-logistic model was used for estimation: $Y=C+(D-C)/(1+\exp[b(\log(x)-\log(I_{50}))])$; ⁺ Values are presented in mean value (standard error); [#] values followed by different letters are significantly (*P*<0.05) different in the column.

The χ^2 test results of F₂ (GDR×GDS) and (GDS×GDR) progeny treated with 840 g ha⁻¹ of glyphosate followed by 560 g ha⁻¹ of dicamba, fit to a resistant:susceptible (9:7) with the *P*-

values of 0.1306 and 0.4497 for F_2 (GDR×GDS), and F_2 (GDS×GDR), respectively. These data confirm that two different genes controlling glyphosate and dicamba resistance in kochia from KS and the segregation pattern also suggest that these two resistance traits are not linked.

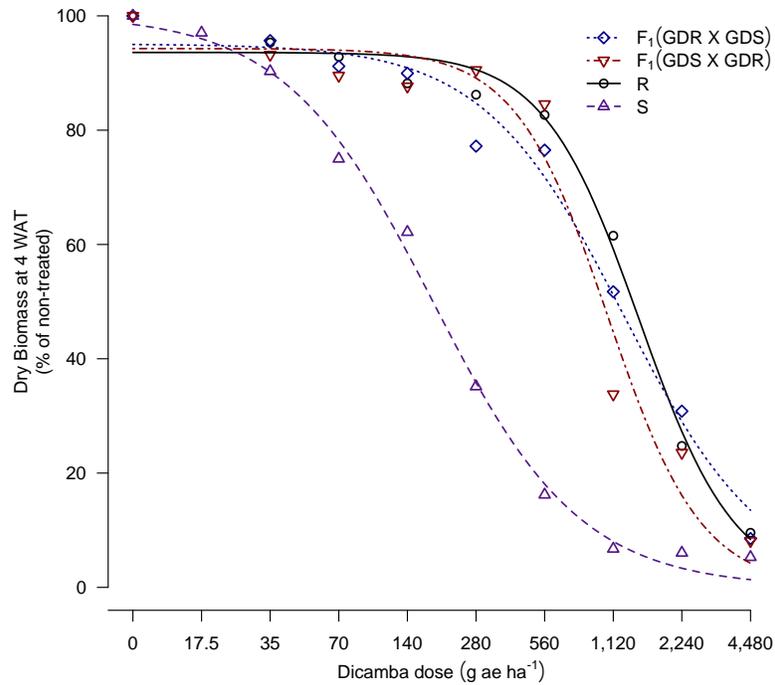


Figure 3.5 Dicamba dose-response of the F_1 progeny of (GDR×GDS) and (GDS×GDR), as well as GDR and GDS kochia.

In conclusion, the F_1 and F_2 analyses of the reciprocal crosses of GDR and GDS kochia demonstrates that the glyphosate resistance in kochia is inherited by a single dominant nuclear gene. Also, this dissertation uncovered for the first time a single semi-dominant trait conferring dicamba resistance in kochia from KS. More importantly, the results of this research also revealed for the first time that the dicamba- and glyphosate-resistant genes in kochia are not linked. Therefore, the resistance to dicamba and glyphosate in kochia appear to have developed, inherited, and spread independently. Although several kochia populations are resistant to both

dicamba and glyphosate, this occurrence is not because of the linkage of the resistance genes.

The high outcrossing nature of kochia, combined with prolific seed production and the tumbling mechanism of seed dispersal, make this species prone to high herbicide selection resulting in the evolution of resistance to multiple herbicides. Consequently, it is essential to use integrated weed management methods to control kochia to contain the spread of glyphosate and/or dicamba resistance, and to prevent further development of resistance to other herbicides.

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Chapter 4 - Management of Dicamba-Resistant Kochia

4.1 Reduced Absorption of Glyphosate and Decreased Translocation of Dicamba Contribute to Poor Control of Kochia (*Kochia scoparia*) at High Temperature

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4.1.1 Abstract

Plant growth temperature is one of the important factors that can influence postemergent herbicide efficacy and impact weed control. Control of kochia (*Kochia scoparia*), a major broadleaf weed throughout the North American Great Plains, often is unsatisfactory when either glyphosate or dicamba are applied on hot summer days. We tested effects of plant growth temperature on glyphosate and dicamba phytotoxicity on two Kansas kochia populations (P1 and P2) grown under the following three day/night (d/n) temperature regimes: T1, 17.5/7.5 °C; T2, 25/15 °C; and T3, 32.5/22.5 °C. Visual injury and above-ground dry biomass data from herbicide dose response experiments indicated greater susceptibility to both glyphosate and dicamba when kochia was grown under the two cooler temperature regimes, i.e. T1 and T2. At T1, the ED₅₀ of P1 and P2 kochia were 39 and 36 g ha⁻¹ of glyphosate and 52 and 105 g ha⁻¹ of dicamba, respectively. In comparison, at T3 the ED₅₀ increased to 173 and 186 g ha⁻¹ for glyphosate and 106 and 410 g ha⁻¹ for dicamba, respectively, for P1 and P2. We also investigated the physiological basis of decreased glyphosate and dicamba efficacy under elevated temperatures. Kochia absorbed more glyphosate at T1 and T2 compared to T3. Conversely, there was more dicamba translocated towards meristems at T1 and T2, compared to T3. Reduced efficacy of dicamba or glyphosate to control kochia under elevated temperatures can be attributed to decreased absorption and translocation of glyphosate and dicamba, respectively. Therefore, it is recommended to apply glyphosate or dicamba when the temperature is low (e.g. d/n temperature at 25/15 °C) and seedlings are small (less than 12 cm) to maximize kochia control.

4.1.2 Introduction

Kochia (*Kochia scoparia* (L.) Schrad.) is one of the most troublesome annual C4 broadleaf weeds in croplands in the Great Plains of North America (Friesen et al., 2009). *Kochia* can emerge early in spring (early March in Kansas) (Dille et al., 2012) before most other spring and summer annual weeds and spring-sown crops and can grow rapidly under cool as well as warm temperatures (Dille et al., 2012; Friesen et al., 2009). Due to its aggressive growth habit, *kochia* can cause huge yield loss in grain crops (Al-Khatib and Peterson, 1999; Friesen et al., 2009). In addition, mature plants of *kochia* accumulate saponins, alkaloids, oxalates, and nitrates, which are toxic to domestic animals (Bokan et al., 2014). More than 30 *kochia* populations across the U.S. have been reported to have evolved resistance to one or more herbicide modes of action (Heap, 2017). Yet, herbicide application is still one of the most effective methods to manage *kochia* in croplands. Weed resistance to herbicide sites of action is evolving at a rapid rate while no new herbicide modes of action has been developed in more than two decades (Duke, 2012). Thus, more efficient use of existing herbicides is vital to maintain their effectiveness in the future.

Poor control of *kochia* in western Kansas has been observed numerous times when glyphosate or dicamba was applied in hot weather (P.W. Stahlman, Personal communication). Incomplete control of *kochia* can accelerate the evolution of glyphosate or dicamba resistance, since long-term or constant exposure to a low/ineffective concentration of a specific herbicide can significantly contribute to the evolution of resistance in weeds (Ashworth et al., 2016; Busi and Powles, 2009).

Several studies have found that the efficacy of commonly used herbicides such as glyphosate, glufosinate, and mesotrione can be affected by temperature. Increased temperature

has altered the efficacy of glyphosate on wild oat (*Avena fatua*) (Adkins et al., 1998), liverseed grass (*Urochloa panicoides*) (Adkins et al., 1998), velvetleaf (*Abutilon theophrasti*) (Zhou et al., 2007), and awnless barnyardgrass (*Echinochloa colona*) (Jordan, 1977). However, only a few studies have investigated the underlying mechanism of altered glyphosate efficacy under different temperature regimes. For instance, Jordan (1977) reported glyphosate controlled bermudagrass (*Cynodon dactylon*) better at high than low temperature because more glyphosate was absorbed and translocated out of the treated leaves. Similarly, Coupland (1983) found elevated basipetal translocation enhanced glyphosate activity at high temperature in couch grass (*Elymus repens*). However, in quackgrass (*Agropyron repens*), Devine et al. (1983) concluded altered efficacy of glyphosate at different temperatures was not due to differential absorption or translocation of the herbicide. Similarly, Friesen and Dew (Friesen and Dew, 1966) reported phytotoxicity of dicamba on tartary buckwheat (*Fagopyrum tataricum*) was not affected when temperature was increased. This study was conducted based on the hypothesis that the temperature can alter absorption and/or translocation of glyphosate or dicamba, thereby affecting kochia control. The objectives of this study were to: a) evaluate the differential efficacy of glyphosate or dicamba at varying temperatures on kochia control and b) investigate the mechanisms underlying the differential efficacy of these herbicides on kochia control.

4.1.3 Materials and Methods

4.1.3.1 Plant Materials and Growth Conditions

Kochia seed was collected from field sites in Pratt County (Brachtenbach, 2015) (Population 1, P1) and Riley County (Population 2, P2), KS in 2012. Because of the short seed longevity of kochia, 5-10 plants from each population annually were grown together in isolation from other kochia and mature seed bulked and stored in dark at 4 °C. Seed from P1 and P2 produced in 2014 were used to conduct glyphosate and dicamba dose response experiments in growth chambers under different temperature regimes (described in detail below). However, only P1 was used to conduct glyphosate and dicamba absorption and translocation experiments at different temperatures.

In 2015, kochia seed of P1 and P2 were germinated in small trays (25 × 15 × 2.5 cm) filled with commercial potting mixture (Pro-Mix Potting-Mix, Premier Tech Horticulture, Ontario, CA). Individual seedlings 2 to 3 cm height were transplanted into plastic pots (6.5 × 6.5 × 9 cm) in a greenhouse on the campus of Kansas State University in Manhattan. The following greenhouse conditions were maintained: 25/20 °C (day/night, d/n) temperatures, 60 ± 10% relative humidity, and 15/9 h day/night photoperiod supplemented with 120 μmol m⁻² s⁻¹ illumination provided with sodium vapor lamps. One week after transplanting, healthy kochia plants (~5 cm height) were transferred to growth chambers that were maintained at different d/n temperatures: T1: 17.5/7.5 °C; T2: 25/15 °C; and T3: 32.5/22.5 °C. Light in all growth chambers was provided by incandescent and fluorescent bulbs delivering 750 μmol m⁻² s⁻¹ photon flux (15/9 h, d/n) at plant canopy level. Due to the unavailability of settings for constant vapor pressure deficit, all the growth chambers were set to maintain 60 ± 10% relative humidity throughout the experiment. Plants were watered daily.

4.1.3.2 Glyphosate- and Dicamba-Dose Response Experiment

4.1.3.2.1 Glyphosate and Dicamba Treatment

Kochia plants were treated with glyphosate (Roundup WeatherMax, Monsanto Co., St. Louis, MO) at dosages of 0, 26.3, 52.5, 105, 210, 420, 840, and 1680 g ha⁻¹ with 2.5% (w/v) ammonium sulfate (AMS) or dicamba (Clarity, BASF Corp., Florham Park, NJ, USA) without AMS at dosages of 0, 17.5, 35, 70, 140, 280, 560, and 1120 g ha⁻¹ when the plants were 10-12 cm height. Herbicides were mixed in water and applied using a bench-type sprayer (Research Track Sprayer, De Vries Manufacturing, Hollandale, MN, USA) equipped with a single moving flat-fan nozzle tip (80015LP TeeJet tip, Spraying Systems Co., Wheaton, IL, USA) delivering 187 L ha⁻¹ at 222 kPa in a single pass at 3.21 km h⁻¹. Following treatment, plants were returned to corresponding growth chambers within 30 min after treatment.

4.1.3.2.2 Visual Injury and Biomass Measurement

Glyphosate- and dicamba-induced injury was rated based on composite visual estimations of growth inhibition, curling, necrosis, and plant vigor on a scale of 0 (no effect) to 100 (plant death). Visual injury ratings were taken at 1, 2, 3, and 4 weeks after treatment (WAT). At 4 WAT, plant stems were cut at soil level and individual plants were placed in separate paper sacks. After oven drying at 60 °C for 72 h, plants were weighed to determine dry biomass.

4.1.3.3 Absorption and Translocation Experiments

Results of the dose response experiments showed that the two kochia populations, P1 and P2, responded similarly to glyphosate and dicamba at each temperature regime. Therefore, the glyphosate or dicamba absorption and translocation experiments were conducted using only one population, i.e. P1.

Prior to conducting the absorption and translocation experiments, we tested whether absorption or translocation of [^{14}C] glyphosate or [^{14}C] dicamba in kochia would be affected by spraying plants with formulated products of either herbicide before [^{14}C] herbicide treatment using the method described by Perez-Jones et al. (2007). Briefly, on six 10-12 cm height kochia seedlings, two newly expanded leaves were marked and wrapped with small pieces of aluminum foil, then the plants were sprayed with formulated product of 840 g ha $^{-1}$ of glyphosate or 560 g ha $^{-1}$ of dicamba using the methods described in section 2.1. After 30 min, when the herbicide droplets dried, the aluminum foil was removed. Likewise, another set of six untreated kochia seedlings of the same size were selected and two newly expanded leaves were marked on these plants as well. On both sets of kochia, the absorption and translocation of [^{14}C] glyphosate or [^{14}C] dicamba were tested under T2 using the method described in detail in sections 4.1.3.3.1 and 4.1.3.3.2. Results (data not shown) indicated that neither absorption nor translocation of [^{14}C] dicamba or [^{14}C] glyphosate was affected by spraying the plants with formulated herbicide. Hence, the absorption and translocation experiments using [^{14}C] glyphosate or [^{14}C] dicamba reported here were not sprayed with formulated herbicide.

Additionally, preliminarily testing of [^{14}C] glyphosate or [^{14}C] dicamba translocation in kochia grown at T2 revealed that less than 0.5% of [^{14}C] glyphosate and only 1.3% of [^{14}C] dicamba was translocated to roots at 72 hours after treatments (HAT). At the same time, 88-95% and 92-96% of [^{14}C] dicamba and [^{14}C] glyphosate, respectively, was recovered from the aboveground parts of kochia. Hence, the translocation of [^{14}C] glyphosate or [^{14}C] dicamba to plant roots was not measured in subsequent experiments.

4.1.3.3.1 Absorption and Translocation of Glyphosate

One milliliter of [¹⁴C] glyphosate working solution with 0.33 kBq μL⁻¹ of radioactivity was prepared by mixing 93.6 μL of [phosphonomethyl-¹⁴C]-Glyphosate water solution (3.7 kBq μL⁻¹, specific activity: 2.04 kBq μg⁻¹, PerkinElmer, Inc., Boston, MA, USA), 9.2 μL of Roundup Weathermax herbicide (Monsanto Co., St. Louis, MO, USA), 73.5 μL of ammonium sulfate (AMS) aqueous solution (34%, w/v) and 823.7 μL of water, which was equivalent to 840 g of glyphosate in a carrier volume of 187 L water with 2.5% (w/v) of AMS.

Kochia seedlings (10-12 cm height) grown under three temperature regimes (as described above) were used. On the upper surface of two newly expanded leaves, 10 μL of [¹⁴C] glyphosate working solution (5 μL per leaf) was applied using Wiretrol® (10 μL, Drummond Scientific Co., Broomall, PA, USA). After 30 min, plants were returned to growth chambers. Plants were harvested at 24, 48 and 72 HAT and separated into treated leaf (TL), tissue above the treated leaf (ATL), and tissue below the treated leaf (BTL). Treated leaves were washed twice with 5 mL wash solution (10% (v/v) ethanol aqueous solution with 0.5% of Tween-20) in 20-mL scintillation vials for 1 min. After adding 15 mL Ecolite-(R) (MP Biomedicals, LLC. Santa Ana, CA, USA), radioactivity in leaf rinsate was measured by using liquid scintillation spectrometry (LSS, Tricarb 2100 TR Liquid Scintillation Analyzer, Packard Instrument Co., Meriden, CT, USA). Plant sections were dried at 60 °C for 72 h and radioactivity in each plant part was quantified by LSS after combusting for three minutes with a biological oxidizer (OX-501, RJ Harvey Instrument, New York, NY, USA).

4.1.3.3.2 Absorption and Translocation of Dicamba

The methods of [¹⁴C] dicamba application and sample collection were the same as described above for the [¹⁴C] glyphosate experiment, except that the 1 mL of [¹⁴C] dicamba

working solution was obtained by mixing 29.3 μL of dicamba-(ring-UL- ^{14}C) ethanol solution (11.4 $\text{kBq } \mu\text{L}^{-1}$, specific activity: 2.87 $\text{kBq } \mu\text{g}^{-1}$, BASF Corp., Florham Park, NJ, USA), 6.4 μL of Clarity herbicide (BASF Corp., Florham Park, NJ, USA) and 964.3 μL of water, which was equal to 560 g of dicamba in a carrier volume of 187 L.

4.1.3.3.3 Data Analysis

The data from absorption and translocation experiments of both herbicides was converted into percentages for further analysis using the following formulas (4.1)-(4.6):

$$\text{Percentage of recovery} = \frac{(R_{\text{rinsate}} + R_{\text{ATL}} + R_{\text{TL}} + R_{\text{BTL}})}{R_{\text{applied}}} \times 100 \quad (4.1)$$

$$\text{Percentage of absorption} = \frac{R_{\text{applied}} - R_{\text{rinsate}}}{R_{\text{applied}}} \times 100 \quad (4.2)$$

$$\text{Percentage of translocation} = 100 - \frac{R_{\text{TL}}}{R_{\text{applied}} - R_{\text{rinsate}}} \times 100 \quad (4.3)$$

$$\text{Percentage in ATL} = \frac{R_{\text{ATL}}}{R_{\text{applied}} - R_{\text{rinsate}}} \times 100 \quad (4.4)$$

$$\text{Percentage in TL} = \frac{R_{\text{TL}}}{R_{\text{applied}} - R_{\text{rinsate}}} \times 100 \quad (4.5)$$

$$\text{Percentage in BTL} = \frac{R_{\text{BTL}}}{R_{\text{applied}} - R_{\text{rinsate}}} \times 100 \quad (4.6)$$

In formulas (4.1)-(4.6), R_{rinsate} is the radioactivity recovered in leaf rinsate; R_{applied} is total amount of radioactivity applied on the plant; R_{ATL} is the radioactivity recovered in tissue above the treated leaf; R_{TL} is the radioactivity recovered in the treated leaf; and R_{BTL} is the radioactivity recovered in tissue below the treated leaf.

4.1.3.4 Experimental Design and Statistical Analysis

Split plot experimental design was used for all experiments. In the glyphosate and dicamba dose response experiments, temperature and herbicide doses were main and subplots, respectively. In absorption and translocation of [¹⁴C] dicamba and [¹⁴C] glyphosate experiments, temperature and harvesting time were the main and subplot, respectively. At least four replicates of each dose were included in both studies and all the experiments were repeated twice in time, and the growth chambers were rotated to avoid pseudo-replication.

In the whole-plant dose response experiments, treatments were arranged in a factorial combination of three levels of growth temperatures (T1, T2, and T3) and different herbicide doses. There was no interaction between experimental runs and treatments; hence, data from the two dose response experiments were pooled for each population prior to analysis. Using the *drc* package (Ritz and Streibig, 2005) in R (v.3.2.1, R Foundation for Statistical Computing, Vienna, Austria), visual injury and dry biomass were subjected to non-linear regression analysis using four parameter log-logistic model (Seefeldt et al., 1995):

$$Y=C+\frac{D-C}{1+\exp[b(\log(x)-\log(I_{50}))]} \quad (4.7)$$

In formula (4.7), *Y* refers to the percentage of control or untreated, *C* is the lower limit, *D* is the upper limit, *b* is the slope, and *I*₅₀ is the dose required for 50% response of plant injury or biomass reduction. This model was used to estimate ED₅₀ (effective dose for 50% control of kochia) and GR₅₀ (effective dose for 50% biomass reduction) values from the visual injury and dry biomass of kochia, respectively.

For experiments involving absorption and translocation, treatments were arranged in a factorial combination of three levels of growth temperatures (T1, T2, and T3) as main factors, and four levels of measurement time (12, 24, 48, and 72 h) as simple factors. There was no

interaction between experimental runs and treatments; hence, data from the two experiments were combined and analyzed by fitting to an asymptotic regression, rectangular hyperbolic or linear model using the method developed by Kniss *et al.* (Kniss et al., 2011) based on *drc* (Ritz and Streibig, 2005) and *qpcR* (Ritz and Spiess, 2008) packages in R program. Furthermore, the bias-corrected Akaike information criterion (AICc) of these three models were compared and the rectangular hyperbolic model (Formula 8) with the lowest AICc value (Kniss et al., 2011) was chosen for analyzing glyphosate or dicamba absorption data. However, none of these three regression models could be used to analyze glyphosate or dicamba translocation data. Therefore, all translocation data were analyzed using two-way ANOVA ($P < 0.05$) in Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

$$Absorption = \frac{A_{max} \times t}{\frac{t_0}{90} \times t + t} \quad (4.8)$$

In formula (4.8), A_{max} is the upper limit (maximum) for absorption of herbicide, t is the time, Absorption is the percentage of absorbed herbicide at time t , and t_{90} refers to the time required to achieve 90% of the maximum absorption.

4.1.4 Results

4.1.4.1 Dose Response of Glyphosate

At 4 WAT, ED₅₀ values for glyphosate on P1 kochia at T1 and T2 were 39 and 68 g ha⁻¹ (Table 4.1; Figures 4.1a), respectively. However, the GR₅₀ values for glyphosate at T1 and T2 were 34 and 42 g ha⁻¹ for this population (Table 4.1; Figures 4.1b). Differences between T1 and T2 were significant (P<0.05) for ED₅₀ but not GR₅₀. However, when d/n temperature was increased to T3, both the ED₅₀ and GR₅₀ increased significantly (P<0.05) to 173 and 171 g ha⁻¹, respectively in P1 kochia. The results of glyphosate dose response on P2 kochia population (Table 4.1; Figures 4.1c and 4.1d) showed similar tendency of growth temperature effects on glyphosate efficacy as described above for P1 kochia. ED₅₀ values for glyphosate on P2 were 36, 68 and 176 g ha⁻¹ at T1, T2, and T3, respectively, whereas the GR₅₀ were estimated as 46, 67, and 187 g ha⁻¹, respectively. Both ED₅₀ and GR₅₀ of glyphosate on P2 increased significantly as growth temperature increased. When the GR₅₀ values were estimated in the four parameters log-logistic model using the raw data of dry biomass, the estimates for other parameters were also generated for glyphosate and listed in Table 4.1. The estimation of *D* values (the upper limit, which represents the dry biomass accumulation of untreated samples) of P1 and P2 were significantly different at T1 and T3 (Table 4.1). In general, the untreated kochia plants grown under cooler temperature (T1) produced three times more biomass than at high temperature (T3; Figures 4.1b, d and 4.2b, d).

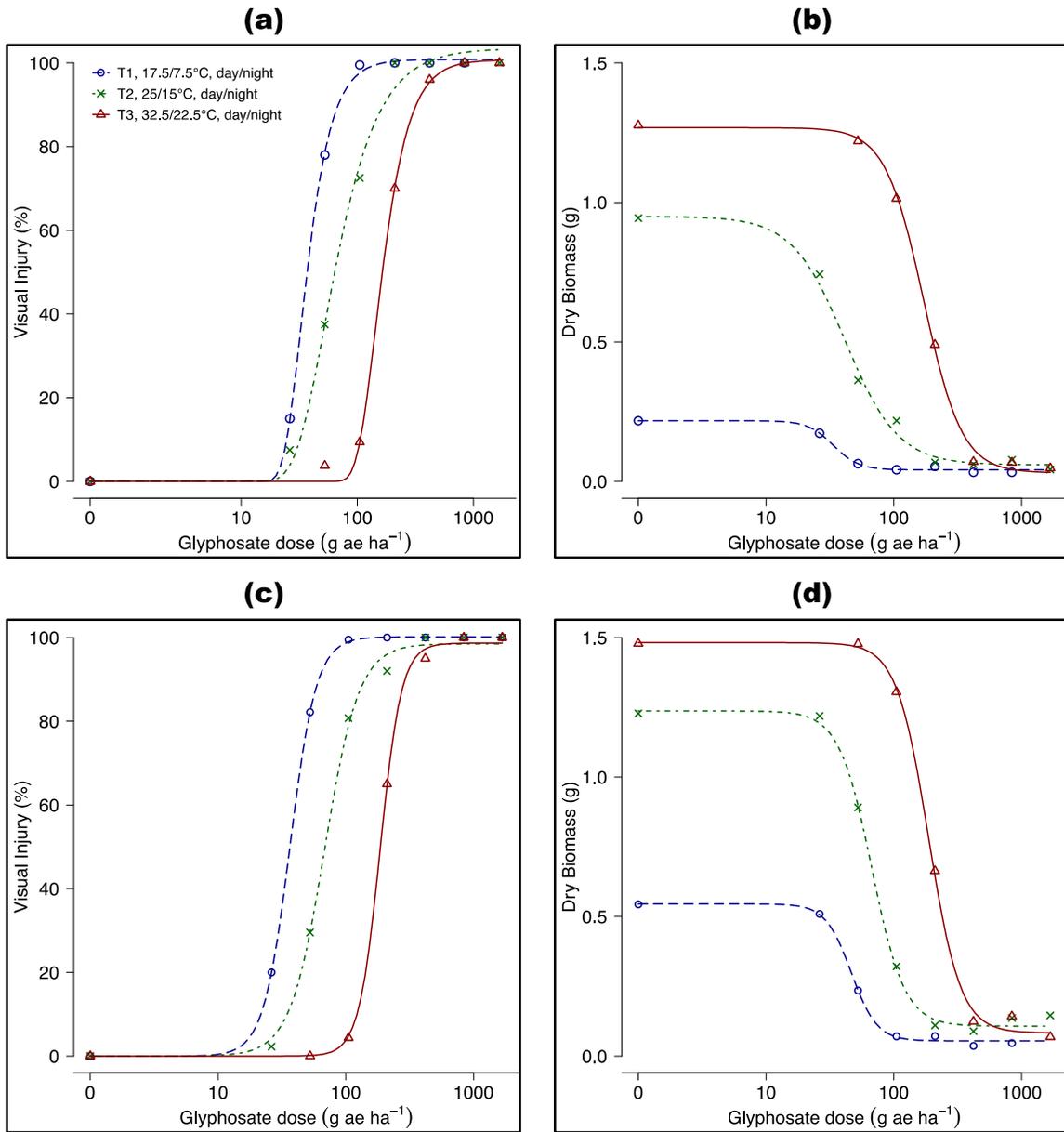


Figure 4.1 Whole-plant glyphosate dose-response of kochia at different temperatures as measured by (a) visual injury (P1), (b) dry biomass (P1), (c) visual injury (P2), (d) dry biomass (P2) at 4 WAT.

Table 4.1 Glyphosate and dicamba dose-response analysis of kochia visual injury and dry biomass under three different temperatures at 4 weeks after treatment*

Herbicide	Kochia population (site of collection)	Temperature (day/night, °C)	ED ₅₀ (g ha ⁻¹)	Parameter estimate (Dry biomass) ⁺			
				GR ₅₀ (g ha ⁻¹)	b	C (g)	D (g)
Glyphosate	P ₁ (Pratt County, KS)	17.5/7.5	39 (2.4) a	34 (5.2) a	4.34 (1.78)	0.04 (0.01)	0.22 (0.02) a
		25/15	68 (4.4) b	42 (11) a	2.10 (1.18)	0.06 (0.08)	0.95 (0.11) b
		32.5/22.5	173 (10) c	171 (55) b	2.90 (2.28)	0.03 (0.20)	1.27 (0.19) bc
	P ₂ (Riley County, KS)	17.5/7.5	36 (2.2) a	46 (1.2) a	4.32 (0.55)	0.05 (0.01)	0.55 (0.01) a
		25/15	68 (6.3) b	67 (1.8) b	3.40 (0.22)	0.11 (0.01)	1.24 (0.02) ab
		32.5/22.5	186 (9.8) c	187 (8.3) c	3.49 (0.43)	0.08 (0.03)	1.48 (0.23) b
Dicamba	P ₁ (Pratt County, KS)	17.5/7.5	52 (2.4) a	21 (15) a	1.96 (3.06)	0.07 (0.06)	0.60 (0.07) a
		25/15	54 (3.4) a	26 (16) a	1.46 (1.94)	0.08 (0.16)	1.28 (0.04) b
		32.5/22.5	106 (6.5) b	73 (19) b	3.99 (5.16)	0.06 (0.26)	1.46 (0.07) c
	P ₂ (Riley County, KS)	17.5/7.5	105 (9.7) a	46 (15) a	0.48 (0.08)	0.24 (0.12)	0.59 (0.05) a
		25/15	167 (34) a	114 (35) a	0.68 (0.37)	0.43 (0.11)	0.95 (0.06) b
		32.5/22.5	410 (36) b	225 (6.3) b	2.76 (0.16)	0.01 (0.02)	1.51 (0.02) c

* Values (mean ± standard error) followed by different letters are significantly (P<0.05) different in each column for each population. ED₅₀ values were calculated using visual injury data. ⁺ The four parameters log-logistic model was used for estimation (see formula 4.7, for details).

4.1.4.2 Dose Response of Dicamba

At 4 WAT, both P1 and P2 kochia showed similar response to dicamba when grown at different temperatures. The ED₅₀ (Table 4.1, Figure 4.2a) of dicamba for P1 kochia was 52, 54, and 106 g ha⁻¹ at T1, T2, and T3, respectively. On the basis of dry biomass, GR₅₀ (Table 4.1, Figure 4.2b) of dicamba for P1 kochia was 21, 26, and 73 g ha⁻¹ at T1, T2, and T3, respectively. Likewise, ED₅₀ of 105, 167, and 410 g ha⁻¹ and GR₅₀ of 46, 114 and 225 g ha⁻¹ at T1, T2, and T3 (Table 4.1, Figure 4.2c and 4.2d), respectively, are estimated for P2 kochia. The efficacy of dicamba on both P1 and P2 decreased when temperature was increased from T2 to T3, but not

from T1 to T2. Also, estimation of the four parameters for dicamba using raw dry biomass data was also determined and listed in Table 4.1. The dry biomass accumulation of untreated samples (*D* values) was significantly different among the three temperature regimes, which indicates temperature has significant effect on growth of kochia.

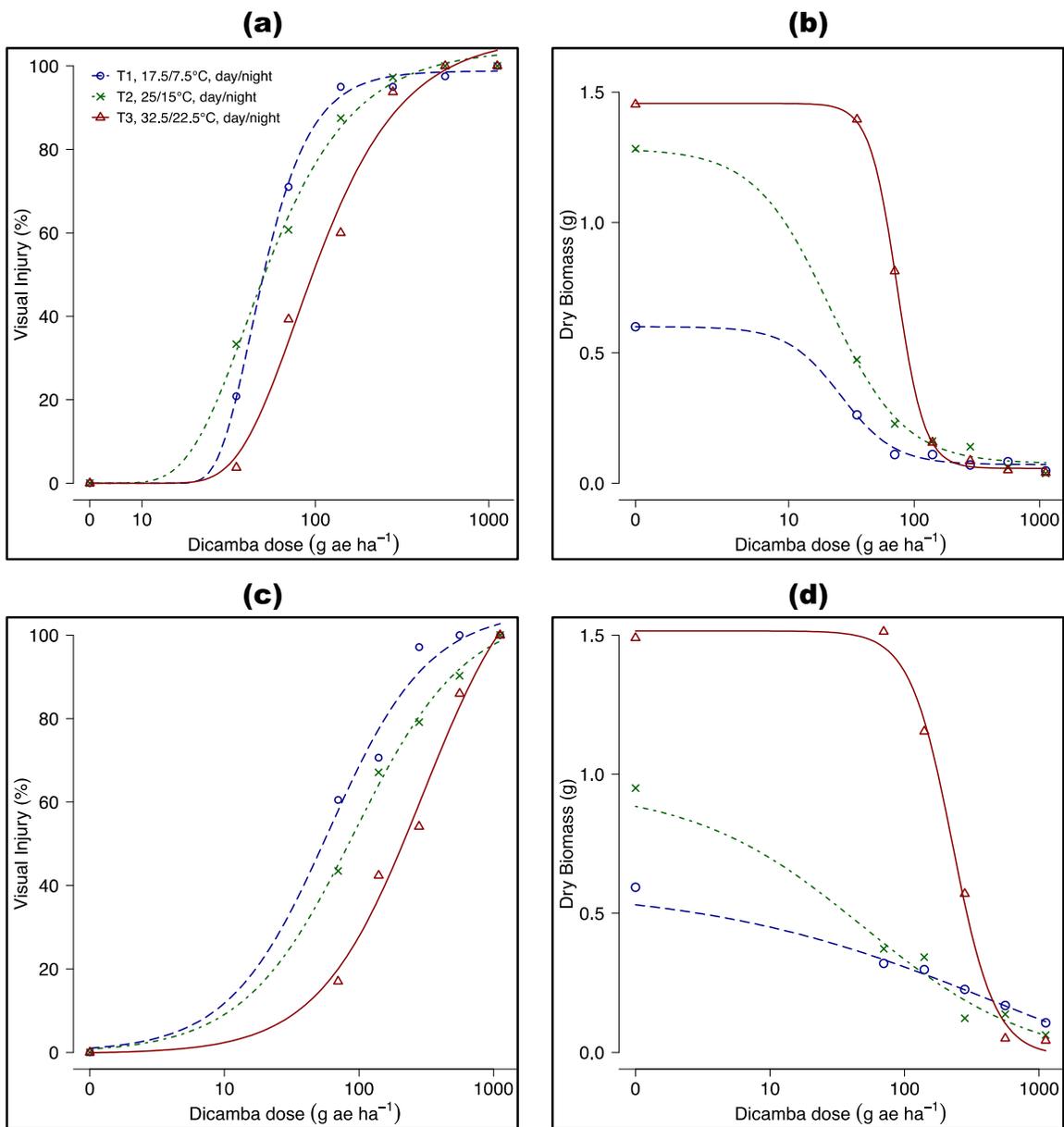


Figure 4.2 Whole-plant dicamba dose-response of kochia at different temperatures as measured by (a) visual injury (P1), (b) dry biomass (P1), (c) visual injury (P2), (d) dry biomass (P2) at 4 WAT.

4.1.4.3 Absorption and Translocation of Glyphosate

Analysis of the data of [¹⁴C] glyphosate absorption/translocation (Table 4.2) indicate the upper limit of absorption of [¹⁴C] glyphosate (A_{max}) as 71, 70, and 41% at T1, T2, and T3,

respectively. When the A_{max} at different temperatures was compared, significantly less [^{14}C] glyphosate was absorbed by kochia at T3 than at T1 or T2. Similarly, analysis of the data by regression model also suggest the time required to achieve 90% of the maximum absorption (t_{90} , Table 4.2) as 188, 144, and 313 hours for T1, T2, and T3, respectively, but the comparison of t_{90} at different temperatures showed the time differences were not significant among the three temperature regimes. Interestingly, regardless of the amount of [^{14}C] glyphosate absorbed, there was no significant difference in the percent of [^{14}C] glyphosate translocated (Figure 4.3b) either to ATL or BTL of kochia grown under any of the temperature regimes tested (Figures 4.3d to 4.3e). Overall, absorption of [^{14}C] glyphosate was significantly reduced when kochia was grown under T3 (Figure 4.3a). However, translocation of [^{14}C] glyphosate in kochia appeared not to be influenced by alterations in temperature (Figure 4.3b). Therefore, reduced absorption of glyphosate may contribute to the lack of control of kochia grown under high temperature.

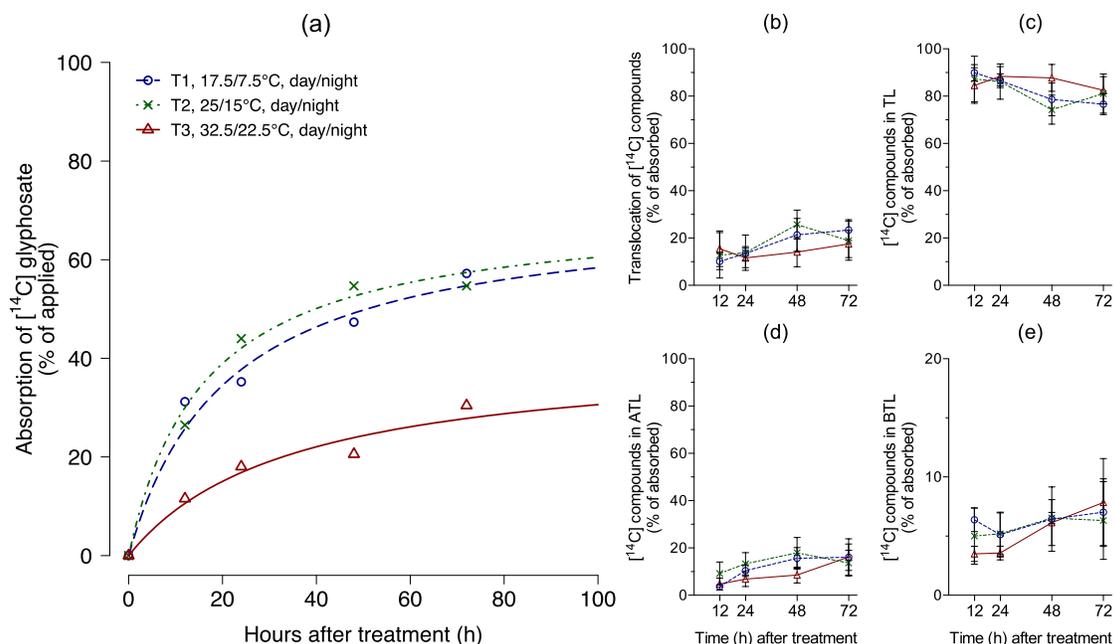


Figure 4.3 [^{14}C] glyphosate absorption (a), translocation (b), retained in treated leaf (c), translocation to above treated-leaf (d), and below treated-leaf (e) at three different temperatures. (* P -value < 0.05, ** P -value < 0.01, *** P -value < 0.001, which indicate the levels of significance within each time points at different temperatures; error bars represent standard error)

Table 4.2 Regression parameter estimates of glyphosate absorption in kochia at different temperatures using rectangular hyperbolic model*

Herbicide	Temperature (day/night, °C)	Parameter estimate	
		Amax	T ₉₀
Glyphosate	17.5/7.5	70.58 (5.77) a	188.03 (44.23) a
	25/15	70.22 (4.32) a	144.55 (29.79) a
	32.5/22.5	41.28 (9.05) b	313.67 (155.89) a
Dicamba	17.5/7.5	98.66 (3.38) a	57.19 (11.73) a
	25/15	97.78 (3.13) a	35.62 (8.86) a
	32.5/22.5	100.0 (3.05) a	47.92 (9.46) a

* see formula (4.8) for the equation of rectangular hyperbolic model.

4.1.4.4 Absorption and Translocation of Dicamba

Similar to absorption of glyphosate, the upper limit of dicamba absorption (A_{max}) and time required to achieve 90% of the maximum absorption (t_{90}) were generated using regression analysis, and the results are shown in Table 4.2. The data suggest A_{max} of 99, 98, and 100%, and t_{90} of 57, 36, and 48 hours for T1, T2, and T3, respectively. However, in contrast to glyphosate, the data of the A_{max} and t_{90} of dicamba was not significantly affected by temperature. While absorption of dicamba increased with time, translocation out of the TL also increased (Figure 4.4b), regardless of temperature. Translocation of [^{14}C] dicamba at 12 and 72 HAT increased from 26 to 47% and 20 to 58% at T1 and T2, respectively (Figure 4.4b). In contrast, at 72 HAT translocation of [^{14}C] dicamba increased from only 6.9 to 21% in kochia grown at T3. This means 20-30% more [^{14}C] dicamba was retained in the TL (Figure 4.4c) of kochia grown at T3, than in kochia grown at T1 or T2. More importantly, at 12 HAT, 16.5 and 16% of [^{14}C] dicamba was translocated to ATL at T1 and T2, respectively, but only 3.2% moved towards meristems in kochia grown at T3 (Figure 4.4d). Conversely, there was no difference ($P>0.05$) in the amount of [^{14}C] dicamba translocated to BTL (Figure 4.4e) in kochia grown at any of the temperature regimes tested. Thus, the poor control of kochia grown under high temperature may be attributed to decreased translocation of dicamba to above treated leaves.

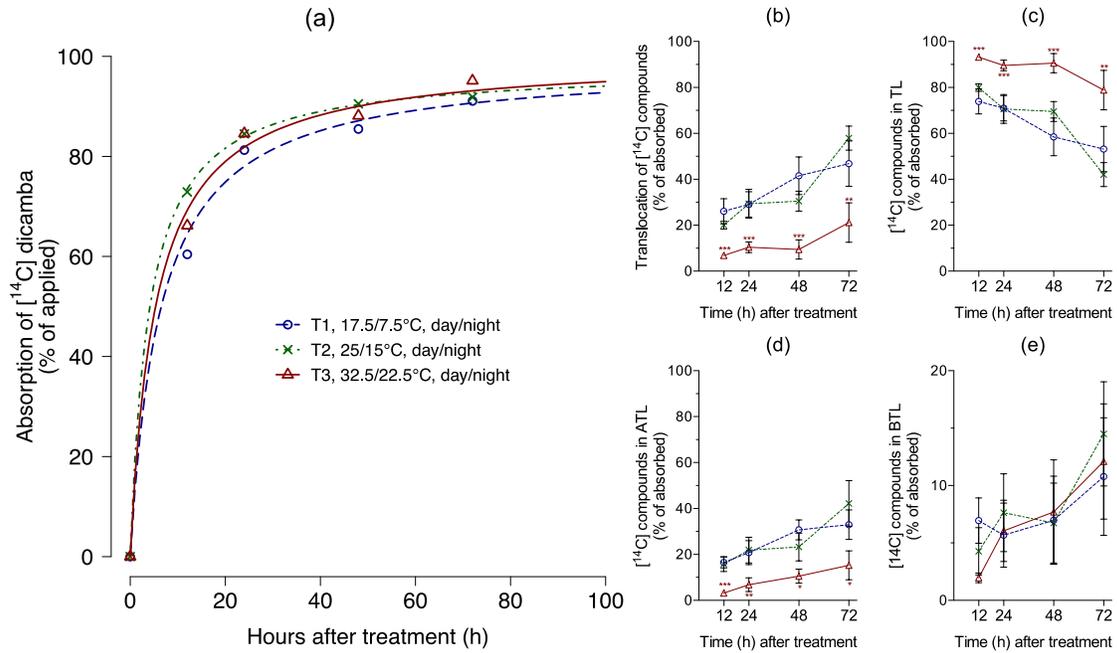


Figure 4.4 $[^{14}\text{C}]$ dicamba absorption (a), translocation (b), retained in treated leaf (c), translocation to above treated-leaf (d), and below treated-leaf (e) at three different temperatures. (** P -value < 0.01, *** P -value < 0.001, which indicate the levels of significance within each time points at different temperatures; error bars represent standard error).

4.1.5 Discussion

In western KS, kochia emerges early- to mid-March and continues into April (Dille et al., 2012) when d/n temperatures are normally about 17.5/7.5 °C (NOAA, 2016). Thereafter, kochia emergence slows down but some seeds can still emerge throughout the growing season. After the major flush of emergence in March to April, kochia starts to grow and accumulates biomass when the d/n temperatures increase to 25/15 °C (NOAA, 2016). POST application of glyphosate or dicamba to control kochia is normally done in mid- to late-June after crop emergence or in July for post-wheat harvest applications when the d/n temperatures are soaring up to 32.5/22.5 °C or higher. This validates selection of these three d/n temperature regimes in this study.

In the dose response experiments, we found the efficacy of glyphosate decreased significantly when the d/n temperatures were increased from 25/15 °C to 32.5/22.5 °C. Similar results were observed for GR₅₀ of glyphosate for P2 kochia (Figure 4.1d), except the GR₅₀ of glyphosate at T1 and T2 on P1 kochia were not significantly different whereas the ED₅₀ of glyphosate on P1 kochia, and both the ED₅₀ and GR₅₀ of glyphosate on P2 kochia were significantly different. These results clearly indicate that plant growth temperature had substantial impact on the efficacy of glyphosate in controlling kochia. Additionally, the nonsignificant estimation of *C* values (data not shown) in the four parameters log-logistic model indicates that kochia (both P1 and P2) accumulated different amounts of dry biomass at all temperatures tested in response to the high rates (lethal rates or higher) of glyphosate or dicamba. This difference in biomass accumulation within each population can be attributed to the inherent genetic variability, which is expected among field populations of kochia. In contrast, in response to any rate of glyphosate or dicamba applied, (except for P1 at T1), the estimation of dry biomass accumulation of untreated samples (*D* values) of both P1 and P2 (Table 4.2) was significant at all

temperature regimes. Specifically, there was significantly higher (about two times) biomass accumulation at T3 than at T1, for P1 and P2 kochia (Figures 4.1b, d and Figures 4.2b, d), which clearly suggests that kochia growth was substantially affected by temperature. The difference in biomass accumulating of kochia at different temperatures may influence the absorption or translocation of herbicides. In general, larger plants are more tolerant to herbicides than the smaller plants. The decreased efficacy of dicamba or glyphosate on kochia grown under high temperatures, possibly because of dilution effect that caused by rapid growth and high biomass accumulation (Kudsk and Kristensen, 1992).

It is known that even with the addition of surfactants, relatively low amounts of applied glyphosate is absorbed by leaves (Brunharo et al., 2015) compared to other systemic herbicides such as dicamba. Our data also show less than 60% of glyphosate absorbed by kochia at 72 HAT (Figure 3a). More importantly, plants typically develop thick, lipophilic cuticles to prevent water loss at high temperature (DeLucia and Berlyn, 1984; Riederer and Schneider, 1990). Therefore, when grown under high temperatures (T3) kochia may develop thicker cuticle, which may have contributed to reduced absorption of glyphosate even when the herbicide was formulated with surfactants (Bradberry et al., 2004). As we observed in our glyphosate dose response experiments, efficacy of glyphosate was decreased at high temperatures, which is highly interrelated with our absorption and translocation data. We conclude the decreased efficacy of glyphosate on kochia at high growth temperature was due to decreased absorption of this herbicide.

In dicamba experiment, GR₅₀ and ED₅₀ dosages for P2 kochia plants were three and four times higher, respectively, compared to GR₅₀ and ED₅₀ dosages for P1 kochia plants (Table 4.1), indicating greater tolerance to dicamba in P2 kochia. Yet, increase in d/n temperature from 25/15

°C to 32.5/22.5 °C reduced the efficacy of dicamba on both P1 and P2 kochia. Based on the dose response results it is evident that efficacy of dicamba on kochia control did not differ when plants were grown under temperature regimes of 17.5/7.5 °C or 25/15 °C; however, efficacy was significantly decreased when they were exposed to 32.5/22.5 °C. In the physiological mechanism study, no difference was found in the amount of [¹⁴C] dicamba absorbed by kochia grown at the temperatures tested in this experiment. However, less [¹⁴C] dicamba was translocated to ATL in kochia grown at T3 than at T1 or T2, while the amount of [¹⁴C] dicamba translocated to BTL was not affected by temperature. Reduced dicamba efficacy on kochia grown at T3 compared to T1 or T2 (Figures 4.2), likely was because of reduced translocation of dicamba to actively growing meristems at T3 (Figure 4b). Dicamba is a systematic herbicide and must be translocated to the meristems (Chang and Born, 1971) to obtain satisfactory weed control. Therefore, the lack of kochia control with dicamba treatment at high temperature (i.e. T3) can be attributed to reduced translocation of this herbicide.

Dicamba absorbed into plant cells can be trapped in phospholipid vesicles due to a hydrophobic interaction between the nonpolar portion of dicamba molecule and the hydrocarbons present in the phospholipid vesicles (Glass, 1988). Since dicamba is predominantly translocated via symplast (Magalhaes et al., 1968), it is prone to get trapped in phospholipid vesicles. It is also known that increased temperature can enhance the strength of hydrophobic interactions of organic molecules (Baldwin, 1986). Therefore, in this study, when dicamba was applied on kochia grown under higher temperature, though the absorption of dicamba was not affected (Figure 4.4a), it is possible that dicamba may have attached to phospholipid vesicles in leaf cells, resulting in lack of movement of this molecule from the site of absorption. Additional study is needed to test this hypothesis.

Furthermore, dicamba is volatile and increased temperature can also accelerate the volatilization of dicamba, regardless of the type of dicamba formulation used (Behrens and Lueschen, 1979). Under field condition, vapor or spray drift of dicamba can cause severe crop damage on soybean (Al-Khatib and Peterson, 1999), tomatoes (Tottman, 1978), and corn (Cao et al., 2010), especially on hot days. Therefore, applying dicamba during periods of high temperature not only reduces kochia control but also increases the risk of off-target crop injury. Dicamba is an auxinic herbicide and sensitive plants show severe injury symptoms (e.g. epinasty, meristem inhibition, and etc.) when treated or exposed to low doses (Grossmann, 2010) of off-target drift. However, dicamba kills susceptible plants slowly. Some of the plants treated with higher than field recommended doses of dicamba in this experiment, although injured severely, still had green tissues at 4 WAT. As a result, it is easy to underestimate dicamba injury symptoms. This can explain the variation in values obtained for ED₅₀ when compared to GR₅₀ at each temperature regime.

4.1.6 Conclusion

Although the mechanisms responsible for the reduced efficacy of dicamba or glyphosate may differ, our results clearly show that kochia is less sensitive to both glyphosate and dicamba when grown under higher temperatures, especially at 32.5 °C. This research provides evidence to support the anecdotal observations made in the field regarding reduced efficacy of herbicides such as dicamba or glyphosate at high temperatures. Therefore, to maximize efficacy of glyphosate and dicamba on kochia and minimize the chances of losing these effective tools for controlling kochia, it will be critical to take action and apply glyphosate or dicamba early in the season after the main flush of kochia emergence when the temperatures are low (e.g. day/night temperature at 25/15 °C, or even lower) and the kochia seedlings are small (less than 12 cm).

4.2 Preemergence Application of Dicamba to Manage Dicamba-Resistant

Kochia (Kochia scoparia)

MANUSCRIPT INFORMATION

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4.2.1 Abstract

Dicamba-resistant crops are being rapidly embraced by growers in the United States to manage glyphosate-resistant and other difficult-to-control broadleaf weeds. However, dicamba resistance in kochia (*Kochia scoparia* (L.) Schrad.), one of the troublesome weeds of the North American Great Plains, is already widespread. Hence, POST application of dicamba may not adequately control kochia. In recent years in the High Plains Region of Colorado, Kansas, and Nebraska, dicamba has been widely applied, often in combination with atrazine or metribuzin, in early spring for PRE control of kochia. However, there is concern this use pattern may increase the selection for dicamba-resistant (DR) kochia. Hence, there is need to understand the efficacy of dicamba applied PRE versus POST for managing DR kochia. A greenhouse study was conducted to test the efficacy of PRE-applied dicamba compared with POST application using both DR and dicamba-susceptible (DS) kochia. Efficacies of PRE-applied dicamba were compared at seeding densities of 300, 600, 900 and 1200 viable seed m⁻². At eight weeks after PRE and four weeks after POST treatment, control of DR kochia seeded at 300 viable seed m⁻² was improved from 10% with 560 g ae ha⁻¹ dicamba applied POST to 94 and 97% with 350 and 420 g ae ha⁻¹ dicamba applied PRE, respectively. However, the efficacy of PRE-applied dicamba was negatively correlated with seed density. When kochia seeding density was increased from 300 to 1200 seed m⁻², the ED₅₀ of PRE-applied dicamba increased from 237 to 705 g ae ha⁻¹ for DR kochia, and from 129 to 361 g ae ha⁻¹ for DS kochia, respectively. Thus, PRE-applied dicamba was effective in controlling the population of DR kochia tested, suggesting that PRE-applied dicamba may still provide substantial control of some DR kochia populations. However, it is not advisable to apply dicamba alone for PRE kochia control.

4.2.2 Introduction

Cropping systems in the North American Great Plains, especially no-till production systems, rely heavily on herbicides for weed control. However, the evolution of resistance to herbicides in many major weeds is constantly threatening agricultural productivity. Preserving the efficacy of herbicides is necessary to maintain the diversity of weed management tools as herbicides with new sites of action have not been released in recent years (Duke, 2012). This is especially true for cropping systems that incorporate herbicide-resistance technologies (Tan et al., 2005).

After being introduced to North America in the 1800s as an ornamental species, kochia (*Kochia scoparia* (L.) Schrad.) quickly became a major problem weed in the Great Plains (Friesen et al., 2009). The rapid evolution and spread of resistance to multiple herbicide modes of action challenges the management of kochia. Currently, there are at least 46 kochia populations with confirmed resistance to herbicides with different sites of action documented in 20 U.S. states, including acetolactate synthase (ALS)-, photosystem (PS) II-, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-inhibitors, and synthetic auxins (Heap, 2017). Because of its outcrossing nature, combined with prolific seed production and a tumbling mechanism of seed dispersal, multiple herbicide-resistant kochia has become a major concern in croplands of the Great Plains, such as Kansas (Varanasi et al., 2015).

Dicamba, one of the most widely used synthetic auxin herbicides, has been an effective herbicide option for kochia control in croplands for decades. Following the widespread occurrence of glyphosate resistance in kochia, dicamba has become one of the key alternatives for kochia management in corn, sorghum, small grains, and other crops. However, several populations of kochia with evolved resistance to dicamba have been reported in Montana, North

Dakota, Idaho, Colorado, Nebraska, and Kansas (Heap, 2017). Recently, Varanasi et al. (2015) reported a single kochia population from Kansas with resistance to four herbicide sites of action, including dicamba. Furthermore, dicamba-resistant crops such as soybean have been rapidly and widely adopted in the U.S., selection pressure will soar from heavier usage. To maintain the efficacy and sustainability of this herbicide, it is essential to develop strategies that enable effective management of dicamba-resistant kochia, especially, for populations that have evolved resistance to other herbicide sites of action.

Soil-applied PRE herbicides have been widely used to provide broad spectrum and prolonged weed control. Herbicide programs that integrate PRE followed by POST applications are widely adopted in different cropping systems (Locke et al., 2002; Norsworthy et al., 2012). Dicamba is registered for PRE use in corn, sorghum, and soybean fields. The efficacy of PRE-applied dicamba on some weed species, such as pigweeds, lambsquarters, horseweed (Bruce and Kells, 1990; Hagood Jr, 1989; Johnson et al., 2010) has been reported, but its efficacy on kochia, especially on dicamba-resistant (DR) populations is not well characterized.

The majority of research on PRE-applied herbicides has focused on the influence of soil properties, such as organic matter content, soil pH, etc. on herbicide efficacy (Blumhorst et al., 1990; Li et al., 2003). However, the influence of weed seed density on the efficacy of PRE-applied herbicide has received little attention. Taylor and Hartzler (2000) reported that increased seed densities of weeds in the seed bank can reduce the efficacy of PRE-applied herbicides. Kochia is a prolific seed producer (Friesen et al., 2009), and seed densities in the field can be highly variable, ranging up to 2600 or more seed m^{-2} (Schweizer and Zimdahl, 1984). In one case, Fay *et al.* (1992) reported kochia seed density of up to 30,000 seed m^{-2} directly underneath an individual mother plant. The impact of kochia seed density on the efficacy of PRE-applied

dicamba has not been previously reported. Therefore, this research was designed with the following objectives: (1) determine the efficacy of PRE vs. POST applied dicamba to control both DS and DR kochia; and (2) evaluate the effect of increasing seed density on the efficacy of PRE-applied dicamba for controlling kochia.

4.2.3 Material and Methods

4.2.3.1 Materials and Growth Conditions

In 2012, kochia seeds were collected from a field in Haskell County, Kansas (37°29'48.5"N, 100°46'53.0"W) (Brachtenbach, 2015). To obtain uniform dicamba-resistant and -susceptible kochia populations, 40 kochia plants from seed collected from the field were self-pollinated to generate 40 lines of first-generation seeds, then 50 plants of each line were planted and treated with dicamba at the label recommended use rate of 560 g ae ha⁻¹. The remaining seeds of a uniformly resistant (survived) line and a uniformly susceptible (killed) line were selected as DR and DS kochia population, respectively, to be used in this study. Our previous research determined the resistance index (the ratio of the effective rate of a herbicide that controls 50% of a resistant biotype relative to a known susceptible biotypes) of the DR kochia compared to the DS kochia was 20 (Ou et al., 2015). Due to short seed longevity of kochia, five DR kochia and five DS kochia plants were grown annually in isolation to prevent outcrossing. Mature seed were bulk collected from the five DR and the five DS kochia separately and stored in the dark at 4 °C to maintain good seed viability. Kochia seed (both DR and DS) harvested in May 2015 were used to conduct the experiments in this study.

Silty loam soil (1.2% OM, pH 8.21), collected near Manhattan, Kansas, USA, was used in this trial. The soil was steam sterilized at 70 °C for 30 minutes in the Hummert's Media Treatment System (Hummert International, Topeka, KS, USA). During the experiments, trays were weekly fertilized from the bottom with Miracle-Gro® water soluble all purpose plant food (1% water solution, N:P:K = 24:8:16, The Scotts Miracle-Gro Company, Marysville, OH, USA). All experiments were conducted in a greenhouse (Department of Agronomy at Kansas State University, Manhattan, KS, USA) using the following environmental conditions: 25/20 °C

(day/night, d/n) temperatures, $60 \pm 10\%$ relative humidity, and 15/9 h d/n photoperiod supplemented with $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ illumination provided using sodium vapor lamps.

4.2.3.2 Germination Test

Since kochia seeds lose viability rapidly, to obtain the exact number of viable seeds required for this study, the germination rate of DR and DS kochia was tested before each experiment using the petri dish method (Chachalis and Reddy, 2000; Everitt et al., 1983). Briefly, three replicates of 50 seeds from each accession were placed on 9.0 cm filter paper (Fisher Scientific, Waltham, MA, USA) in 10 cm plastic Petri dishes (Phyto Technology Laboratories, Shawnee Mission, KS, USA) with 5 ml distilled water. Petri dishes were sealed with Parafilm (Bermis company, Inc. Oshkosh, WI, USA) and incubated in a dark room at $25 \text{ }^{\circ}\text{C}$. Seed germination was determined when a visible radicle protrusion occurred at 1 week after incubation (WAI). Germination percentage for each population was calculated as $G = (n_1/50+n_2/50+n_3/50)/3$, where n_1 , n_2 , and n_3 are the number of germinated kochia seeds at 1WAI in Petri dish #1, 2, and 3, respectively. The number of seeds planted (N) in each tray was adjusted according to the germination rate G using the following formula: $N = D \times A / G$, where D is the planting density of viable seeds, and A is the surface area of soil in trays (0.0375 m^2).

4.2.3.3 Efficacy of PRE- vs. POST-Applied Dicamba on DR and DS Kochia

Seedling trays ($25 \times 15 \times 15 \text{ cm}$) were filled with steam-sterilized soil to a depth of 14 cm and were watered to saturation from the bottom. Kochia seeds were spread at a density of 300 viable seed m^{-2} on top of the soil and covered with a thin layer of fine soil particles. Trays were randomly assigned to untreated, PRE or POST treatments. After planting, dicamba (Clarity[®]; BASF Corp., Florham Park, NJ, USA) at 280, 350, and 420 g ae ha^{-1} was applied to the soil surface in PRE treatment trays using a bench-type sprayer (Research Track Sprayer, De Vries

Manufacturing, Hollandale, MN, USA) equipped with a single moving flat-fan nozzle tip (80015LP TeeJet tip, Spraying Systems Co., Wheaton, IL, USA) at a height of 30.5 cm, delivering 187 L ha⁻¹ at 222 kPa in a single pass at 3.21 km h⁻¹. To incorporate the herbicide into the soil, water equivalent to 0.2 mm rain was applied to the soil surface using the same sprayer. In the trays assigned for POST treatment, 560 g ha⁻¹ of dicamba (Clarity®)(field recommended rate) was applied to 10- to 12-cm height DR and DS kochia at four weeks after planting.

The number of plants that survived in each tray was recorded at one through eight weeks after planting (WAP). At eight WAP, all plant material above the soil surface in each tray was harvested and placed in paper sacks. After drying at 60 °C for 72 h in an oven, plant material was weighed to determine dry biomass. Each treatment was replicated four times, and the experiment was repeated twice.

4.2.3.4 Efficacy of PRE-Applied Dicamba to Control Kochia at Different Seeding Densities

To determine the efficacy of PRE-applied dicamba on DR or DS kochia, the same methods described previously in the PRE vs POST experiments were used with the exception that four planting densities (300, 600, 900, or 1200 viable seed m⁻²) were used instead of one, and the dicamba (Clarity®) rates changed to 0, 140, 280, 560, and 1120 g ha⁻¹. Treatments were replicated four times, and the experiment was repeated three times.

4.2.3.5 Data Analysis

A completely randomized design was used in both studies. Data for the number of plants that survived and dry biomass were analyzed using two-way ANOVA ($P < 0.05$) in Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). In the efficacy of PRE- vs. POST-applied dicamba on DR and DS kochia experiment, data for the number of surviving plants and dry

biomass were analyzed using two-way ANOVA ($P < 0.05$) in Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). Treatments were arranged in a factorial combination of four levels of seed densities and four dicamba rates. There was no interaction between experimental runs and treatments; hence, the data from the three experimental runs were pooled together for the statistical analyses. The data from the study determining the efficacy of PRE-applied dicamba for kochia control at different seed densities were analyzed using the drc package (Ritz and Streibig, 2005) in R (v.3.2.1, R Foundation for Statistical Computing, Vienna, Austria)(Team, 2015). The number of surviving plants and dry biomass data were subjected to nonlinear regression analysis using four-parameter log-logistic model (Seefeldt et al., 1995):

$$Y = C + (D - C)/(1 + \exp[b(\log(x) - \log(I_{50}))]) \quad (4.9)$$

where Y refers to the response variable (either number of surviving seedlings or dry biomass), C is the lower limit, D is the upper limit, b is the slope, and I_{50} is the rate (x) required for 50% response of the number of plants survived or biomass reduction. This model was used to estimate ED_{50} (dicamba rate required for 50% stand loss of kochia plants) and GR_{50} (dicamba rate required for 50% biomass reduction) values from the number of plants that survived and dry biomass of kochia, respectively.

4.2.4 Results and Discussion

4.2.4.1 Efficacy of PRE- vs. POST-Applied Dicamba on DR and DS Kochia

Control

Based on the number of plants that survived dicamba treatment, PRE application of ≥ 280 g ha⁻¹ of dicamba controlled more than 99% of DS kochia plants (Fig. 4.5a). In comparison, the POST-applied labeled rate of 560 g ha⁻¹ of dicamba controlled 85% of the same kochia accession. PRE-applied dicamba at 280, 350, and 420 g ha⁻¹ provided 75, 94, and 97% control of DR kochia, respectively, whereas POST application of 560 g ha⁻¹ dicamba controlled only 10% of the DR accession. Similar results were observed for dry biomass measurements (Fig. 4.5b). Specifically, PRE application of dicamba at 280, 350, and 420 g ha⁻¹ reduced DS kochia 94, 99 and 100%, respectively, whereas the POST application of 560 g ha⁻¹ reduced biomass 82%. Also, at least 98% DR kochia biomass reduction was achieved using PRE-applied dicamba at ≥ 350 g ha⁻¹, while only 5% biomass reduction resulted from applying 560 g ha⁻¹ dicamba POST.

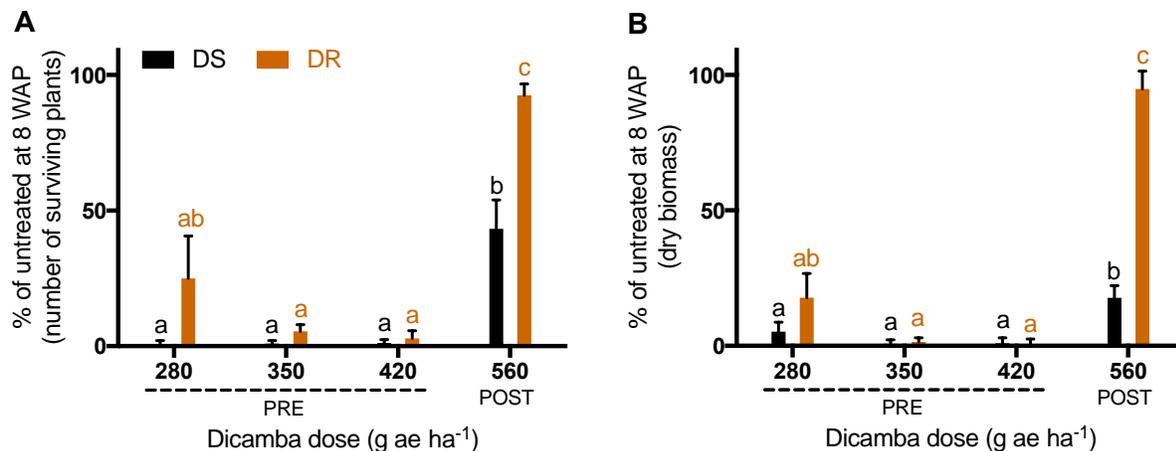


Figure 4.5 Percentage of dicamba-susceptible (DS) and dicamba-resistant (DR) kochia control using PRE (preemergence)-applied dicamba at kochia density of 300 viable seed m⁻² compared with POST (postemergence)-applied dicamba. (A) number of surviving plants, (B) dry biomass. The bars marked by different letters are significantly (Fisher's protected LSD, *P*-value ≤ 0.05) different in each panel.

Reduction in biomass accumulation indicates that plants that survived PRE-applied dicamba were severely injured, as less biomass accumulated during the eight-week growing period following application. This is notable considering the low level of plant competition as most kochia seedlings were killed at germination or early seedling stage. This reduction in biomass would likely also reduce per plant seed production (Wilson et al., 1995). Nevertheless, other management methods should be incorporated or followed to stop the kochia seedbank renewal and reduce selection for further development of dicamba resistance in kochia populations.

4.2.4.2 Efficacy of PRE-Applied Dicamba on Kochia at Different Seeding

Densities

The efficacy of PRE-applied dicamba on both DS and DR kochia negatively correlated with seeding density. The estimated values of ED₅₀ (dicamba rate required for 50% stand loss of kochia plants) using the four-parameter log-logistic model (Eqn. 1) is listed in Table 4.3, and model fitted curves for number of surviving plants are shown in Fig. 2a. Regardless of the kochia accession, the ED₅₀ values of dicamba increased with increasing seed density, except the ED₅₀ values were not different between densities of 600 and 900 viable seed m⁻² for DS kochia (Table 4.3). The ED₅₀ for dicamba on DS kochia increased from 129 to about 206 g ha⁻¹ when seeding density increased from 300 to 600 or 900 viable seed m⁻² and further increased to 361 g ha⁻¹ when seeding density increased to 1200 viable seed m⁻² (Table 4.3). For DR kochia, the trend of ED₅₀ changes with increasing seeding densities was similar to DS kochia, but all ED₅₀ values were significantly different at all four levels of seeding density tested. Specifically, when seeding density increased from 300 to 600, 900, and 1200 viable seed m⁻², ED₅₀ increased from 235 to 356, 468 and 699 g ha⁻¹, respectively (Table 4.3).

Table 4.3 Estimated values of ED₅₀ and GR₅₀ using the nonlinear regression analysis of four parameter log-logistic model*.

Kochia	Density	ED ₅₀ [#]	GR ₅₀ [#]	RI ⁺ (ED ₅₀)	RI ⁺ (GR ₅₀)
	viable seed m ²	g ae ha ⁻¹			
Dicamba-susceptible	300	129 (6) a	130 (2) a	-	-
	600	206 (12) b	264 (6) b	-	-
	900	229 (16) b	244 (6) b	-	-
	1200	427 (72) c	461 (20) c	-	-
Dicamba-resistant	300	235 (11) a	250 (11) a	1.8	1.9
	600	356 (25) b	404 (14) b	1.7	1.5
	900	468 (21) c	677 (19) c	2.0	2.8
	1200	699 (73) d	1266 (106) d	1.6	2.7

* model: $Y = C + (D - C)/(1 + \exp[b(\log(x) - \log(I_{50}))])$; # ED₅₀ (dicamba rate required for 50% stand loss of kochia) and GR₅₀ (dicamba rate required for 50% biomass reduction) values were estimated using the number of surviving plants data and dry biomass data, respectively. Values in parenthesis are standard error. Different letters indicate a significant difference among the seed densities within each population (Fisher's protected LSD, P -value ≤ 0.05). + RI, Resistance indices, the ratio of the effective rate that control 50% of dicamba-resistant (DR) kochia to the effective rate that control 50% of dicamba-susceptible (DS) kochia.

A similar relationship between seeding densities and GR₅₀ were observed. As seeding density increased from 300 to 600 and 1200 viable seed m⁻², GR₅₀ values of DS kochia increased from 130 to 264 and 461 g ha⁻¹; and GR₅₀ values of DR kochia increased from 250 to 404 and 1266 g ha⁻¹, respectively (Table 4.3, Figure 4.6b).

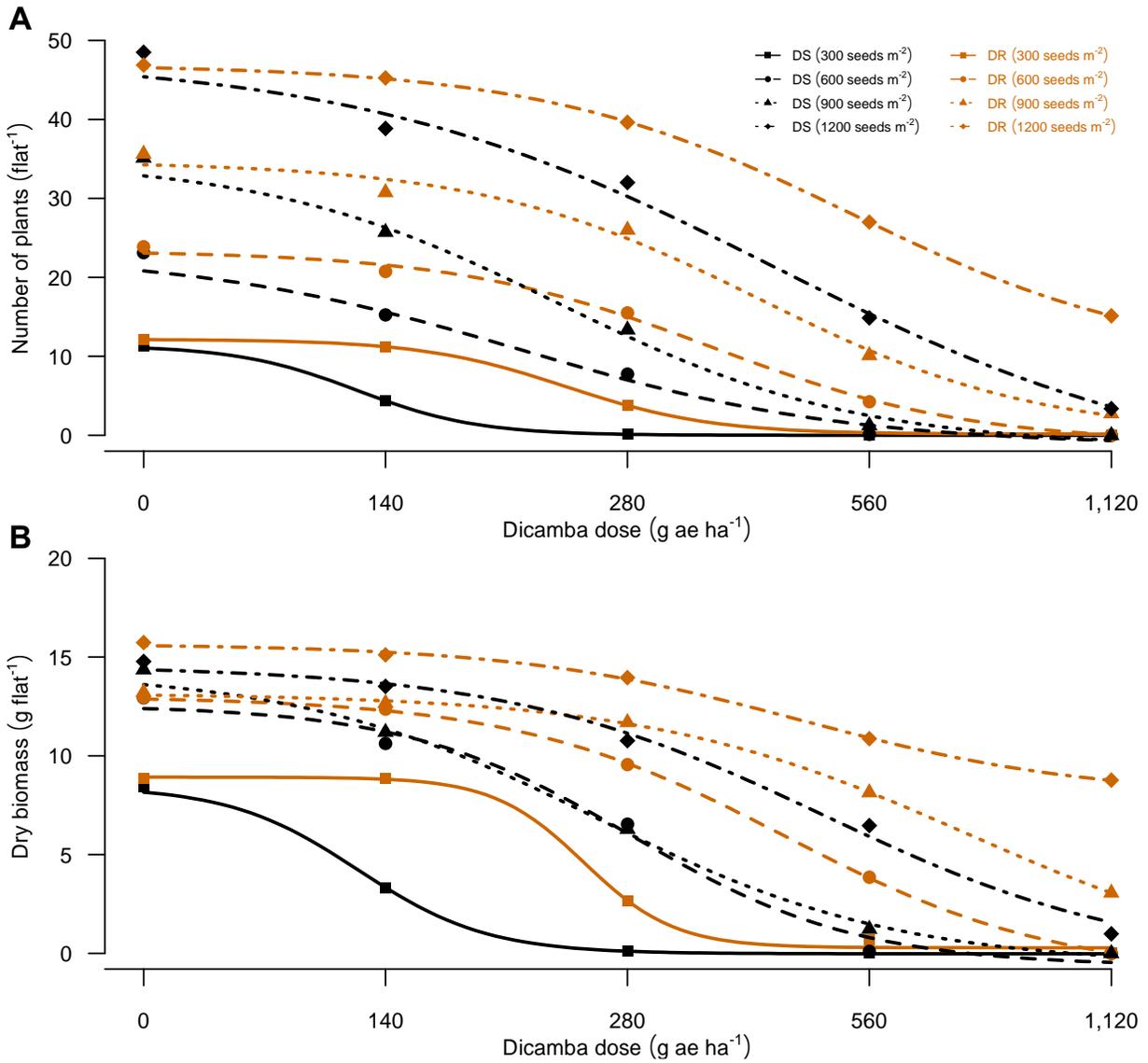


Figure 4.6 Dose-response of kochia to PRE (preemergence)-applied dicamba at different seed densities as measured by (a) number of surviving plants, (b) dry biomass. (Non-linear regression model: $Y=C+(D-C)/(1+\exp[b(\log(x)-\log(I_{50}))])$).

The resistance indices indicate significantly higher rates of dicamba were required to achieve 50% control of DR than DS kochia at each seeding density, either to achieve 50% stand loss of kochia or to achieve 50% biomass reduction (Table 4.3). Resistance indices of DR kochia compared to DS kochia in response to PRE-applied dicamba were 1.8, 1.7, 2.0, and 1.6 at

seeding densities of 300, 600, 900 and 1200 viable seed m^{-2} , respectively (Fig. 4.6a). Similarly, the calculated resistance indices of DR kochia compared to DS kochia using dry biomass data were 1.5, 1.9, 2.8, and 2.7 for seeding densities of 300, 600, 900 and 1200 viable seed m^{-2} , respectively (Fig. 4.6b). The resistance indices ranged from 1.5 to 2.8, suggesting 1.5- to 2.8-times more PRE-applied dicamba was required to provide 50% control of DR kochia than DS kochia. However, previous research showed the resistance index of this DR kochia accession compared to the same DS kochia (used in this research) in response to POST-applied dicamba was 20 (Ou et al., 2015), requiring 20 times more dicamba POST to control the DR kochia accession than the DS kochia accession. The resistance index decreased drastically from 20 for POST-applied dicamba to 1.5 to 2.8 for PRE-applied dicamba.

The ability of prolific seed production and tumbling mechanism of seed spread makes the seed bank of kochia highly variable (Friesen et al., 2009). According to the results of tested seed densities in this study, 560 g ha^{-1} of PRE-applied dicamba could possibly provide consistent kochia control in fields where seed densities range from 1 to 1200 viable seed m^{-2} if no dicamba resistance is observed in the field. At the same time, the 560 g $ae ha^{-1}$ of PRE-applied dicamba may still provide consistent control if the DR kochia seed density is less than 600 viable seed m^{-2} in the field. While there is currently no label recommended rate for kochia control using PRE-applied dicamba; it is critical to apply the full recommended rate of dicamba with complete coverage to ensure effective and consistent control of kochia throughout fields. Moreover, to reduce the selection of higher level of dicamba resistance in kochia populations, it is essential to practice the best weed management practices (Norsworthy et al., 2012) by adding other effective herbicides with different modes of action in the PRE application of dicamba.

The outcome of this research suggests that PRE application of dicamba may still be a feasible option to control kochia, even with widespread dicamba resistance in kochia on the Great plains. Although, no single specific tool can be the silver bullet to solve the worldwide problem of herbicide resistance. PRE application of dicamba should always accompanied with other effective management tools to maintain its sustainability.

**4.3 Reduced Translocation of Glyphosate and Dicamba in Combination
Contributes to Poor Control of *Kochia scoparia*: Evidence of Herbicide
Antagonism**

MANUSCRIPT INFORMATION

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4.3.1 Abstract

Kochia (*Kochia scoparia* (L.) Schrad.) is a troublesome weed across the Great Plains of North America. Glyphosate and dicamba have been used for decades to control kochia. Due to extensive selection, glyphosate- and dicamba-resistant (GDR) kochia have evolved in the USA. Herbicide mixtures are routinely used to improve weed control. Herbicide interactions if result in an antagonistic effect can significantly affect the management of weeds, such as kochia. To uncover the interaction of glyphosate and dicamba when applied in combination in kochia management the efficacies of different doses of glyphosate plus dicamba were evaluated under greenhouse and field conditions using GDR and a known glyphosate- and dicamba-susceptible (GDS) kochia. The results of greenhouse and field studies suggest that the combination of glyphosate and dicamba application controlled GDS, but glyphosate alone provided a better control of GDR kochia compared to glyphosate plus dicamba combinations. Furthermore, investigation of the basis of this response suggested glyphosate and dicamba interact antagonistically and consequently, the translocation of both herbicides was significantly reduced resulting in poor control of kochia. Therefore, a combination of glyphosate plus dicamba may not be a viable option to control GDR kochia.

4.3.2 Introduction

Due to wide adoption of no-till agriculture, crop production in North American Great Plains is highly dependent on the use of herbicides (Bridges, 1994). However, because of the extensive and prolonged use of herbicides with the same site of action, a number of weed species evolved resistance to herbicides, which is one of the major threats to sustainable crop production.

Additionally, rapid adoption of herbicide-resistant crops, is also contributing to the evolution of herbicide resistance in weeds due to lack of herbicide rotation, thereby increased selection pressure (Powles and Preston, 2006).

Kochia scoparia (L.) Schrad. (kochia), a member of Chenopodiaceae, was introduced to North America for ornamental purpose (Friesen et al., 2009). Soon after its introduction, kochia has become highly invasive in crop fields and rangelands (Friesen et al., 2009). Because of its ability to tolerate drought, salinity, cold, and prolific seed production as well as having a tumbling mechanism of seed dispersal (Culpepper et al., 2017; Dille et al., 2017; Friesen et al., 2009; Westra et al., 2017), kochia has turned out to be one of the worst weeds in North American Great Plains. Without timely management, kochia can cause huge yield loss in crops such as corn, sorghum, wheat, soybean, and sugarbeet (Friesen et al., 2009; Waite et al., 2013). In the last three decades, the situation has further exacerbated as a result of rapid and wide spread of resistance to acetolactate synthase-, photosystem II-, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)- inhibitors, and synthetic auxins (Heap, 2017). The management of kochia, especially those populations that are herbicide-resistant has become an important component in cropping systems in North American Great Plains.

Combinations of multiple herbicides have been used to control a broader spectrum of weeds (Wrubel and Gressel, 1994), and to minimize the amount of herbicides applied

(Blackshaw et al., 2006; Zhang et al., 2013). More importantly, tank mixing of different herbicides has been recommended to delay the evolution of herbicide resistance in weed populations (Behrens et al., 2007; Jasieniuk et al., 1996; Vencill et al., 2012). However, not all herbicides can be used in combinations, due to incompatibility or antagonism between certain herbicide chemical groups (Zhang et al., 1995). Combinations of glyphosate plus dicamba have been recommended as burndown application before planting no-till cotton to control horseweed (*Conyza canadensis* L.) (Waggoner et al., 2011). In Kansas, glyphosate plus dicamba are usually sprayed together to manage wide spectrum of monocot and dicot weed species including kochia, especially after evolution and spread of glyphosate resistance in weed populations since 2007 (Heap, 2017). However, inconsistent results when glyphosate plus dicamba combination were applied indicate that the interaction between these two herbicides can be species specific. For instance, O'Sullivan and O'Donovan (1980) reported antagonistic interaction between glyphosate and dicamba, resulting in decreased phytotoxicity of glyphosate on monocot crops such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and weeds, like wild oats (*Avena fatua* L.). Flint and Barrett (1989) reported that combinations of glyphosate plus dicamba could reduce the efficacy of glyphosate on johnsongrass (*Sorghum halepense* L.) due to reduced uptake and translocation. In contrast, Eubank *et al.* (2008) suggested addition of dicamba to glyphosate can increase control of horseweed from 70% to over 90%. However, the response of kochia to glyphosate and dicamba combination is not known. When applied in combination, if these two herbicides exhibit an antagonistic interaction, this can result in poor control of kochia, consequently, may accelerate the evolution of glyphosate and/or dicamba resistance because of exposure to less effective doses of these herbicides (Ashworth et al., 2016; Busi and Powles, 2009), which in turn can weaken the herbicide options for the management of this weed. The

glyphosate- and dicamba-resistant (GDR) kochia populations have been reported and wide spread throughout the US Great Plains, including Kansas (Heap, 2017). The significance of application of glyphosate and dicamba in combination on kochia control is not known. Hence, it is important to investigate the interaction of glyphosate and dicamba on kochia, to evaluate if use of these herbicides in combination help better control of this weed. Moreover, because growers are expected to rapidly embrace the new glyphosate- and dicamba-resistant crops, it is vital to understand the interaction of glyphosate and dicamba in kochia to maintain the sustainability of the herbicide resistant crops in the kochia infested regions. Therefore, this research was conducted with the following objectives: 1) test the efficacy of glyphosate plus dicamba combinations on GDR kochia in greenhouse and field conditions; and 2) investigate the physiological interaction of glyphosate and dicamba in GDR kochia using radioactive labelled herbicides, by comparing with a known glyphosate- and dicamba-susceptible (GDS) kochia.

4.3.3 Materials and Methods

In 2012, kochia seed were collected from a field in Haskell County, Kansas (37°29'48.5"N, 100°46'53.0"W). kochia plants generated from these seeds were self-pollinated by keeping the plants in isolation from other kochia plants and upon maturity seed were harvested separately from ten plants. One hundred seedlings were generated separately from seed harvested from above 10 plants. When plants reached 10-12 cm height, 50 plants each were treated with a label recommended field rate of glyphosate (840 g ae ha⁻¹) or dicamba (560 g ae ha⁻¹). In response to glyphosate or dicamba treatment, all the progeny of a single plant tested that were completely killed, these were selected as glyphosate- and dicamba-susceptible (GDS) kochia. The remaining seed harvested from the same GDS mother plant was used in all experiments in this research. Likewise, all the progeny of single plant tested that survived glyphosate or dicamba treatment, were selected as glyphosate- and dicamba-resistant (GDR) kochia. Also, the rest of the seed harvested from the same GDR mother plant was used in this research.

Greenhouse experiments were conducted in weed science greenhouse attached to the Department of Agronomy at Kansas State University, Manhattan, Kansas, United States. The following greenhouse conditions were maintained: 25/20°C (day/night, d/n) temperatures, 60 ± 10% relative humidity, and 15/9 h d/n photoperiod supplemented with 120 µmol m⁻² s⁻¹ illumination provided with sodium vapor lamps. The physiological studies were conducted in growth chambers maintained at following conditions: 25/15°C d/n temperature, 60 ± 10% relative humidity, and 15/9 h d/n photoperiod, light was provided by incandescent and fluorescent bulbs delivering 750 µmol m⁻² s⁻¹ photon flux at plant canopy level.

4.3.3.1 Glyphosate- and Dicamba-Dose Response of GDR and GDS kochia

GDR and GDS kochia seeds were germinated in trays ($25 \times 15 \times 2.5$ cm) filled with commercial potting mixture (Pro-Mix Potting-Mix, Premier Tech Horticulture, Ontario, CA). Individual seedlings at 6-leaf stage were transplanted into plastic pots ($6.5 \times 6.5 \times 9$ cm) containing the same type of soil and kept in the same greenhouse as above. When the kochia seedlings were 10-12 cm height, they were treated with glyphosate (Roundup WeatherMax[®], Monsanto Co., St. Louis, MO, USA) at 0, 52.5, 105, 210, 420, 840 (label recommended field, i.e. 1X dose), 1680, and 3360 g ae ha⁻¹ with 2.5% (w/v) ammonium sulfate (AMS) or dicamba (Clarity[®], BASF Corp., Florham Park, NJ, USA) without AMS at 0, 70, 140, 280, 560 (label recommended field, i.e. 1X dose), 1120, 2240, 4480, and 8960 g ae ha⁻¹.

The above treatments were applied as follows. Herbicides were mixed according to the labels and applied using a bench-type sprayer (Research Track Sprayer, De Vries Manufacturing, Hollandale, MN, USA) equipped with a single moving even flat-fan nozzle tip (8002E TeeJet tip, Spraying Systems Co., Wheaton, IL, USA) delivering 187 L ha⁻¹ at 207 kPa in a single pass at 4.85 km h⁻¹. At four weeks after herbicide treatment (WAT), glyphosate- and dicamba-induced visual injury was rated based on composite visual estimation of growth inhibition, epinasty (downward curling of plant parts), necrosis, and plant vigor on a scale of 0 (no effect) to 100 (plant death). Plant were clipped off at soil level at 4 WAT and individual plants were placed in separate paper sacks. Dry biomass data was obtained by weighing after oven dried at 60 °C for 72 h.

4.3.3.2 GDR and GDS Kochia Response to Glyphosate plus Dicamba Combinations under Greenhouse Conditions

GDR and GDS kochia seedlings were produced as described above. When plants reached 10-12 cm height in the greenhouse, 19 combinations of low to high doses of glyphosate plus dicamba (Table 4.5) were applied (as described above) on both GDR and GDS kochia to test their efficacy. At 4 WAT, the number of dead plants was recorded.

4.3.3.3 GDR and GDS Kochia Response to Glyphosate plus Dicamba Combinations under Field Conditions

Field studies were conducted in 2015 and 2016 at Western Kansas Agricultural Research Center - Hays, Kansas, United States. To minimize the effect of herbicide residue, experimental plots were set in different fields next to each other in 2015 and 2016. The GPS coordinates of the field in 2015 and 2016 were 38°51'44.72" N, 99°20'8.76" W, and 38°51'44.72" N, 99°19'59.34" W, respectively.

In 2015, GDS and the GDR kochia seeds were germinated in Planters Pride™ plastic greenhouse kit (72 cells, The HC Companies, Middlefield, OH, USA) in the greenhouse. When the seedlings reached 3-4 cm, twenty plants of either GDS or GDR kochia seedlings were transplanted by hand into each field plot of 3 ×3 m. The field was sprinkler irrigated daily. After the seedlings were recovered from transplantation and reached to 10-12 cm height, five treatments including 2100 g ae ha⁻¹ of glyphosate, 1400 g ae ha⁻¹ of dicamba, 2100 g ae ha⁻¹ of glyphosate mixed with 700 g ae ha⁻¹ of dicamba, 2100 g ae ha⁻¹ of glyphosate mixed with 1400 g ae ha⁻¹ of dicamba, and a non-treated control were used and designated as 2.5G, 2.5D, 2.5G+1.25D, 2.5G+2.5D, and non-treated, respectively (Table 4.6), were applied using a CO₂-pressured backpack sprayer with a 2.74 m boom that was equipped with six TTI110015 tip at 275 kPa with a spray volume of 140 L ha⁻¹ by walking at 4.8 km h⁻¹ approximately. Visual injury data (as described above) were collected at 1, 2, 3, and 4 WAT.

In 2016, the experiment was repeated using the same method as described above for in the year 2015, except GDS and GDR kochia seeds were directly planted into the 3 m×3 m plots, and hand weeding was implemented to remove other weeds.

4.3.3.4 Absorption and Translocation of [¹⁴C] Glyphosate vs. [¹⁴C] Glyphosate plus Dicamba Combination in GDR and GDS Kochia

In a previous research, we reported absorption or translocation of both [¹⁴C] glyphosate and [¹⁴C] dicamba in kochia were not affected by spraying plants with formulated herbicides prior to application of [¹⁴C] labeled compounds (Ou et al., 2016). In the same research, we also found that less than 5% of dicamba and glyphosate translocated to below ground tissue of kochia (Ou et al., 2016). Therefore, in this study, the kochia plants were not treated with herbicide formulations prior to application of [¹⁴C] labeled dicamba or glyphosate, and also the radioactivity in below ground parts of kochia was not tested.

One mL of [¹⁴C] glyphosate working solution (*hot-G*) with 0.33 kBq μL^{-1} of radioactivity was prepared by mixing 93.6 μL of [phosphonomethyl-¹⁴C]-glyphosate (3.7 kBq μL^{-1} , specific activity: 2.04 kBq μg^{-1} , PerkinElmer, Inc., Boston, MA, USA), 20.5 μL of Roundup WeatherMax[®] herbicide, 73.5 μL of AMS aqueous solution (34%, w/v) and 812.4 μL of water, which was equivalent to 2100 g of glyphosate in a carrier volume of 187 L water with 2.5% (w/v) of AMS. Another mL of [¹⁴C] glyphosate plus dicamba combination solution (*hot-GD*) with 0.33 kBq μL^{-1} of radioactivity, which was equivalent to 2100 g of glyphosate and 1400 g of dicamba in a carrier volume of 187 L water with 2.5% (w/v) of AMS, was prepared by mixing 93.6 μL of [phosphonomethyl-¹⁴C]-glyphosate, 20.5 μL of Roundup WeatherMax[®] herbicide, 15.6 μL of Clarity[®] herbicide, 73.5 μL of AMS aqueous solution (34%, w/v), and 796.8 μL of water.

Radioactive herbicides were applied on GDR and GDS kochia as follows. kochia seedlings were grown in a growth chamber and when plants were 10-12 cm height, two newly expanded leaves were marked. Ten μL of *hot-G* or *hot-GD* solution (5 μL per leaf) was applied using Wiretrol[®] (10 μL , Drummond Scientific Co., Broomall, PA, USA). Thirty minutes after herbicide application, plants were returned to the same growth chamber. Plant tissue was harvested at 24, 72 and 168 hours after treatment (HAT) and dissected into treated-leaves (TL), tissue above the treated leaves (ATL), and tissue below the treated leaves (BTL). TL were gently washed twice with 5 mL of 10% (v/v) aqueous ethanol solution with 0.5% of Tween-20 for one minute. Radioactivity in the rinsate was quantified using liquid scintillation spectrometry (LSS, Beckman Coulter LS6500 Multipurpose Scintillation Counter, Beckman Coulter, Inc. Brea, CA, USA) after adding 15 mL of Ecolite-(R) (MP Biomedicals, LLC. Santa Ana, CA, USA). Plant parts (TL, ATL, and BTL) were dried in oven at 60 °C for 72 h and combusted for three minutes with a biological oxidizer (OX-501, RJ Harvey Instrument, New York, NY, USA), the radioactivity in each plant part was quantified by LSS.

4.3.3.5 Absorption and Translocation of [¹⁴C] Dicamba vs. Glyphosate plus [¹⁴C]

Dicamba Combination in GDR and GDS Kochia

The methods of application and sample collection of both [¹⁴C] dicamba (*hot-D*) and [¹⁴C] dicamba plus glyphosate (*hot-DG*) combination solution were the same as described above for glyphosate vs. glyphosate plus dicamba combination experiments, except that the 1 mL of *hot-D* working solution (equal to 1400 g of dicamba in a carrier volume of 187 L) with 0.33 kBq μL^{-1} of radioactivity was obtained by mixing 29.3 μL of dicamba-(ring-UL-¹⁴C) ethanol solution (11.4 kBq μL^{-1} , specific activity: 2.87 kBq μg^{-1} , BASF Corp., Florham Park, NJ, USA), 15.4 μL of Clarity[®] herbicide (BASF Corp., Florham Park, NJ, USA), 73.5 μL of AMS, and 881.8 μL of

water; and the 1 mL of [¹⁴C] dicamba and glyphosate (*hot*-DG) combination solution (equal to 1400 g of dicamba and 2100 g of glyphosate in a carrier volume of 187 L) with 0.33 kBq μL⁻¹ of radioactivity was obtained by mixing 29.3 μL of dicamba-(ring-UL-¹⁴C) ethanol solution, 15.4 μL of Clarity[®] herbicide, 20.8 μL of Roundup WeatherMax[®] herbicide, 73.5 μL of AMS, and 881.8 μL of water.

4.3.3.6 Phosphor Image Analysis of Glyphosate or Dicamba vs. Glyphosate plus Dicamba Combination in GDR and GDS Kochia

Due to the nature of kochia leaves, i.e. long and narrow, and to maximize the sensitivity of the phosphor image analysis, new working solutions of [¹⁴C] glyphosate, [¹⁴C] dicamba, [¹⁴C] glyphosate with formulated dicamba combination, and [¹⁴C] dicamba with formulated glyphosate combination containing 3.3 kBq μL⁻¹ of radioactivity, (denoted by *hot*-G', *hot*-D', *hot*-GD', and *hot*-DG', respectively), were prepared using the same method as described above.

GDR and GDS kochia seeds were germinated in trays filled with the commercial potting mixture as described above. Individual seedlings 2 to 3 cm height were transplanted into plastic pots (6.5 × 6.5 × 9 cm) that filled with silica sand (Granusil[®] Handy Sand, Fairmount Santrol, Sugar Land, TX, USA) and rinsed in 1% (w/v) of Miricle-Gro water soluble All Purpose Plant Food (N:P:K=24:8:16, Scotts Miracle-Gro Products Inc. Marysville, OH, USA) and kept in growth chamber. When the kochia seedlings were 6-8 cm height (10-12 cm height plants were not selected, because the plants were taller to manipulate for phosphor image analysis), they were treated with 1 μL droplet of *hot*-G', *hot*-D', *hot*-GD', and *hot*-DG' on one newly expanded leaf. At 24, 72, and 168 HAT, kochia plants were gently uprooted, and the roots were washed with water carefully. Then, the whole plant was washed twice with 10 mL of 10% (v/v) ethanol aqueous solution with 0.5% of Tween-20 for 1 minute, and then pressed using a handmade plant

press(Lacey et al., 2001) and dried at 60°C for 72 h. The pressed kochia plants were exposed to BAS-IP MS 2040 E Multipurpose Standard Storage Phosphor Screen (GE Healthcare Life Sciences, Pittsburgh, PA, USA) for 44 h (the *hot-G'* and *hot-GD'* treated plants) or 24 h (the *hot-D'* and *hot-DG'* treated plants), and the screen was read using Bio-Rad molecular imager FX (Bio-Rad Laboratories, Inc. Hercules, CA, USA).

4.3.3.7 Experimental Design and Data Analysis

Split plot design was used in the experiment of glyphosate and dicamba dose response on GDR and GDS kochia. Kochia population and herbicide dose were main- and subplot, respectively. Treatments were arranged in a factorial combination with GDR and GDS kochia and different herbicide doses. No interaction between experimental runs was observed; hence, data from the repeated experiments were pooled prior to analysis. Then, visual injury and dry biomass data were subjected to non-linear regression analysis using four parameter log-logistic model (Seefeldt et al., 1995) in R (v.3.2.1, R Foundation for Statistical Computing, Vienna, Austria) with the *drc* package (Ritz and Streibig, 2005).

$$Y=C+(D-C)/(1+\exp[b(\log(x)-\log(I_{50}))]) \quad (1)$$

In Eqn 1, Y refers to the percentage of untreated, C and D are the lower limit and upper limit of the data, respectively, b is the slope, and I_{50} is the dose required for 50% response of visual injury or biomass reduction, which was used to estimate ED_{50} (effective dose for 50% control of kochia) and GR_{50} (effective dose for 50% biomass reduction) values from the visual injury and dry biomass data, respectively.

Split plot experimental design was also used in greenhouse screening experiments and efficacy study of different glyphosate plus dicamba combinations in field conditions. Kochia population and rate of herbicide combination were the main- and subplot, respectively. Data

from the repeated experiments were pooled prior to analysis due to no interaction between experimental runs was found. Two-way analysis of variance was performed in GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) using Bonferroni's multiple comparisons test (P -value <0.05).

Randomized completely block design with a split plot and subsampling was used in the absorption and translocation of radioactive herbicides, where, kochia population and herbicide (herbicide alone or combination) were the whole-plot treatment factors, the sampling times as split-plot factor, and the dissected plant parts as the subsampling factor. No interaction between experimental runs was found. Therefore, data of the total amount of absorption, percent of absorption, translocation amount, percent of translocation from repeated experiments were pooled prior to analysis and analyzed separately using SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA) using the MIXED procedure.

Split plot experimental design was used in the phosphor imaging analysis, in which main- and subplot were kochia population and sample harvesting time, respectively. Phosphor images were processed in Quantity One software 4.6.9 (Bio-Rad Laboratories, Inc. Hercules, CA, USA), and the plant photos were processed in GNU Image Manipulation Program 2.8.20 (GIMP development team, <https://www.gimp.org>)

At least four biological replicates (individual plants) of each treatment, dose or harvesting time were included for each experiment, and all the experiments were repeated twice in time.

4.3.4 Results

4.3.4.1 Glyphosate- and Dicamba-Dose Response of GDR and GDS Kochia

In all the experiments the label recommended doses of glyphosate and dicamba used were 840 and 560 g ae ha⁻¹, respectively. Analyses of herbicide dose response data (Table 4.4) suggested that the GDR kochia were resistant to glyphosate at 840 g ae ha⁻¹. For instance, ED₅₀ (effective dose for 50% control of kochia) and GR₅₀ (effective dose for 50% biomass reduction) of glyphosate for GDR kochia were 978 and 835 g ae ha⁻¹ (Table 4.4), respectively, which were close to or higher than 840 g ae ha⁻¹. On the other hand, the values of ED₅₀ and GR₅₀ of glyphosate for GDS kochia were significantly lower at 518 and 391 g ae ha⁻¹ (Table 4.4), respectively. Based on ED₅₀ or GR₅₀ estimates, the GDR kochia was found to be twice more resistant to glyphosate than GDS (Table 4.4).

The results of dicamba dose-response suggested higher level of resistance to dicamba than glyphosate in GDR kochia. The ED₅₀ of dicamba for GDR and GDS kochia were 1259 and 72 g ae ha⁻¹, respectively, whereas, GR₅₀ estimates were 2529 and 106 g ae ha⁻¹, respectively (Table 4.4). These data suggest that the GDR kochia is 20 times more resistant to dicamba than the GDS kochia.

Table 4.4 Estimated values of ED₅₀ and GR₅₀ of glyphosate and dicamba in kochia using the nonlinear regression analysis of four parameter log-logistic model*.

Herbicide	Kochia	ED ₅₀ [#]	GR ₅₀ [#]
-----g ae ha ⁻¹ -----			
Glyphosate	GDR	978 (6)	835 (26)
	GDS	518 (38)	391 (28)
	Resistance indices ⁺	1.9(0.2)	2.2(0.3)
Dicamba	GDR	1259 (310)	2529 (438)
	GDS	72 (4)	106 (15)
	Resistance indices ⁺	18(6)	25(8)

* model: $Y=C+(D-C)/(1+\exp[b(\log(x)-\log(I_{50}))])$; ED₅₀ (effective dose for 50% control of kochia) and GR₅₀ (effective dose for 50% biomass reduction) values were estimated using the number of plants data and dry biomass data, respectively. # Values in parenthesis are standard error. + resistant level of GDR kochia population comparing to GDS population using ED₅₀ or GR₅₀ values.

4.3.4.2 GDR and GDS Kochia Response to Glyphosate plus Dicamba

Combinations under Greenhouse Conditions

GDR and GDS kochia response to herbicide combinations is presented as percentage of non-treated (%) in Table 4.5. The field recommended rate of dicamba (560 g ae ha⁻¹, Treatment (Trt) 1) and 1400 g ae ha⁻¹ (Trt 2, 2.5 times of field recommended rate) controlled 0 and 14% of GDR and 82% and 88% of GDS kochia, respectively. When half of the recommended field rate of glyphosate (420 g ae ha⁻¹) was mixed with 350 g ae ha⁻¹ of dicamba (Table 4.5, Trt 3), it provided 22 and 47% of GDR and GDS kochia control respectively. However, when 420 g ae ha⁻¹ of glyphosate was mixed with 700 g ae ha⁻¹ of dicamba, it only provided 13 and 50% control of GRD and GDS kochia, respectively.

In general, glyphosate alone without mixing with dicamba showed the best control of both GDR and GDS kochia, compared to the combinations containing the same dose of glyphosate. For example, 840 g ae ha⁻¹ of glyphosate (Trt 5) had 45 and 95% control of GDR and GDS kochia, respectively. It rendered more control of GDR kochia than glyphosate and dicamba combinations (Trt 6, 7, and 8); and had similar control of Trt 9, which was mixed with 560 g ae ha⁻¹ of dicamba. Also, the 840 g ae ha⁻¹ of glyphosate (Trt 5) controlled GDS kochia more effectively than Trt 6 and 7 (Table 4.5).

When Trt 10 to 14 were compared, 1260 g ae ha⁻¹ of glyphosate alone (Trt 10) rendered higher or similar control of the combinations that contain 140 to 1400 g ae ha⁻¹ of dicamba with the same amount of glyphosate. In the case of combinations with 2100 g ae ha⁻¹ of glyphosate, the results suggest that 2100 g ae ha⁻¹ of glyphosate (Trt 15) alone controlled the 95% of GDR kochia, which is higher than Trt 16, 17, and 19 that were mixed with 140, 280 and 1400 g ae ha⁻¹ of dicamba, respectively. When 700 g ae ha⁻¹ of dicamba was mixed with 2100 g ae ha⁻¹ of glyphosate, the control of kochia was similar to the application of 2100 g ae ha⁻¹ of glyphosate alone. However, all the combinations containing 2100 g ae ha⁻¹ of glyphosate rendered similar control of GDS kochia except the Trt 19, which was mixed with 1400 g ae ha⁻¹ of dicamba and rendered only 91% control of GDS kochia.

Table 4.5 The treatments and efficacies (4 WAT) of glyphosate plus dicamba combinations on GDR and GDS kochia in greenhouse conditions.

Trt	Dicamba dose -----g ae ha ⁻¹ -----	Efficacy*	
		GDR -----%-----	GDS
Glyphosate at 0 g ae ha ⁻¹			
1	560	0 A	82(7) a
2	1400	14(3) B	88(5) a
Glyphosate at 420 g ae ha ⁻¹			
3	350	22(8) A	47(8) a
4	700	13(5) A	50(9) a
Glyphosate at 840 g ae ha ⁻¹			
5	0	45(3) A	95(1) a
6	70	31(2) B	80(3) b
7	140	30(3) B	82(2) bc
8	280	30(5) B	91(3) ac
9	560	43(3) A	87(2) ab
Glyphosate at 1260 g ae ha ⁻¹			
10	0	51(8) AB	96(1) a
11	140	40(5) AB	92(3) ab
12	280	57(9) A	96(1) a
13	840	24(6) B	90(4) ab
14	1400	48(7) AB	85(2) b
Glyphosate at 2100 g ae ha ⁻¹			
15	0	95(3) A	100 a
16	140	56(5) C	97(2) ab
17	280	59(5) C	92(3) ab
18	700	93(4) AB	96(3) ab
19	1400	79(2) C	91(1) b
non-treated	0	0	0 0

* Means of visual injury (n=20), and the values in parentheses are standard error. The values followed by different letters are significantly (P -value<0.05) different among the treatments that contain the same dose of glyphosate within each population according to the Bonferroni's multiple comparisons test.

4.3.4.3 GDR and GDS Kochia Response to Glyphosate plus Dicamba

Combinations under Field Conditions

The results of kochia control at 4 weeks after treatment (WAT) with combinations of glyphosate and dicamba are presented in Table 4.6. Similar to the results obtained under greenhouse conditions, the treatment 2.5G (2.5 times of glyphosate at label recommended dose) controlled 98% of GDR kochia, which is better than when treated with dicamba alone (e.g. 2.5D, 2.5 times of dicamba at label recommended dose). On the other hand, all the treatments with 2100 g ae ha⁻¹ of glyphosate, including the 2.5G, 2.5G+1.25D (1.25 times of dicamba at label recommended dose), and 2.5G+2.5D, rendered 100% control of GDS kochia. But, the treatment 2.5D that contained 1400 g ae ha⁻¹ of dicamba only provided 84% GDS kochia control at 4 WAT, which is significantly less than the other treatments.

Table 4.6 The treatments and efficacies (4 WAT) of glyphosate plus dicamba combinations applied on GDR and GDS kochia in field conditions.

Trt*	Herbicide doses		Efficacy [#]	
	Glyphosate	Dicamba	GDR	GDS
	-----g ae ha ⁻¹ -----		-----%-----	
2.5G	2100	0	98(2) A	100 a
2.5D	0	1400	18(5) B	84(5) b
2.5G+1.25D	2100	700	83(6) C	100 a
2.5G+2.5D	2100	1400	78(4) C	100 a
non-treated	0	0	0	0

* 2.5G, 2.5D, and 1.25D represents 2.5 times of glyphosate at label recommended dose (840 g ae ha⁻¹), 2.5 times of dicamba at label recommended dose (560 g ae ha⁻¹), and 1.25 times of dicamba at label recommended dose for kochia control, respectively. # Means of visual injury (n=8), and the values in parentheses are standard error (n=8). The values followed by different letters are significantly (*P-value*<0.05) different among the four treatments within each population according to the Bonferroni's multiple comparisons test.

4.3.4.4 Absorption and Translocation of [¹⁴C] Glyphosate and [¹⁴C] Glyphosate plus Dicamba Combination in GDR and GDS Kochia

In both GDR and GDS kochia, more [¹⁴C] glyphosate was absorbed when [¹⁴C] glyphosate was mixed with dicamba (*hot-GD*) than [¹⁴C] glyphosate was applied alone (*hot-G*) at 24 hours after treatment (HAT), e.g., 30.2 and 47.9% of [¹⁴C] glyphosate was absorbed at 24 HAT when *hot-G* and *hot-GD* was applied on GDR kochia, respectively (Fig. 4.7a). However, there was no difference in herbicide absorption at 72 and 168 HAT between glyphosate or glyphosate plus dicamba combination in both GDR (Fig. 4.7a) and GDS (Fig. 4.7b) kochia. In both GDR and GDS kochia, the [¹⁴C] glyphosate translocation pattern suggest that more [¹⁴C] glyphosate was retained in treated leaves (TL) when *hot-GD* was applied than *hot-G* alone (Fig. 4.7c and 4.7d).

The difference in translocation of glyphosate was observed at both 72 and 168 HAT in GDR kochia (Fig. 4.7c), and it was also found at 168 HAT in GDS kochia (Fig.1d). This indicates less [¹⁴C] glyphosate was translocated away from TL when glyphosate was mixed with dicamba in both GDR and GDS kochia (Fig. 4.7c and 4.7d). Also, less [¹⁴C] glyphosate was translocated to plant parts above-treated leaf (ATL) in both GDR (Fig. 4.7e) and GDS (Fig. 4.7f) kochia at 168 HAT when glyphosate was mixed with dicamba than when was applied by itself. Especially, less translocation of [¹⁴C] glyphosate occurred in plant parts below-treated leaf (BTL) with *hot-GD* than *hot-G*. At all the time points tested, including 24, 72, and 168 HAT, significantly less [¹⁴C] glyphosate was translocated to BTL in both GDR (Fig. 4.7g) and GDS (Fig. 4.7h) kochia with *hot-GD* than *hot-G*. Phosphor image analysis also confirmed these results, with less [¹⁴C] glyphosate translocated to shoots, leaves, and roots when glyphosate was mixed with dicamba in both GDR (Fig. 4.8b) and GDS (Fig. 4.8f) kochia, compared to glyphosate alone in GDS (Fig. 4.8a) and GDR (Fig. 4.8e), respectively.

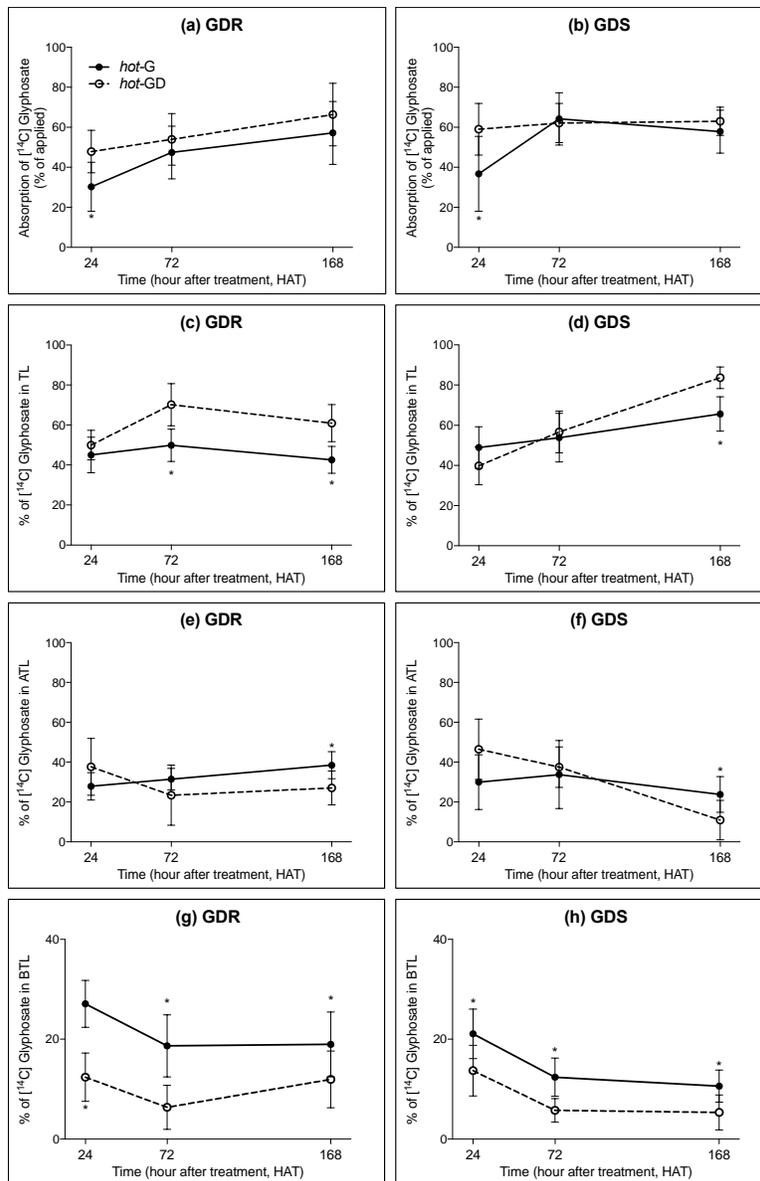


Figure 4.7 [¹⁴C] glyphosate absorption (a and b), retained in treated leaves (TL, c and d), translocated to above treated leaf part (ATL, e and f) and below treated leaf part (BTL, g and h) in glyphosate- and dicamba-resistant (GDR, a, c, e, and g), and glyphosate- and dicamba-susceptible (GDS, b, d, f, and h) kochia when glyphosate alone (*hot-G*, solid line), and glyphosate plus dicamba combination (*hot-GD*, broken line) was applied. (* *P*-value < 0.05, which indicate the levels of significance at each time point for different herbicide treatments; error bars represent standard deviation, n=8)

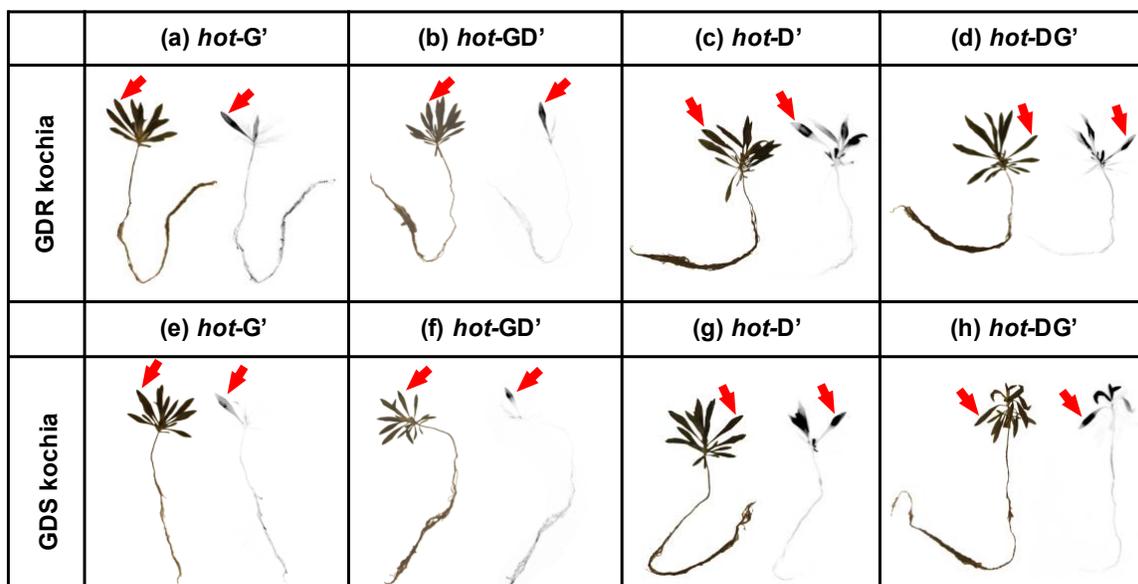


Figure 4.8 Phosphor images of [^{14}C] glyphosate (*hot-G'*, a and e), [^{14}C] glyphosate plus dicamba combination (*hot-GD'*, b and f), [^{14}C] dicamba (*hot-D'*, c and g), and [^{14}C] dicamba plus glyphosate combination (*hot-DG'*, d and h) in glyphosate- and dicamba-resistant (GDR, a, b, c, and d) and glyphosate- and dicamba-susceptible (GDS, e, f, g, and h) kochia at 168 hours after treatment (HAT). Each plant is shown in RGB image (left) and phosphor image (right). In phosphor image, the darker color represents more radioactivity. Arrow points at the treated leaf on each plant where radioactive herbicide was applied.

4.3.4.5 Absorption and Translocation of [^{14}C] Dicamba and Glyphosate plus [^{14}C]

Dicamba Combination in GDR and GDS Kochia

When glyphosate was mixed with [^{14}C] dicamba (*hot-DG*), more [^{14}C] dicamba was absorbed at 24 HAT in GDR kochia (Fig. 4.9a) than when [^{14}C] dicamba (*hot-D*) applied alone, but this difference was not observed at later time points tested (i.e. 72 and 168 HAT) in GDR (Fig. 4.9a) or in GDS kochia (Fig. 4.9b). Translocation data indicate more [^{14}C] dicamba was retained in TL at 168 HAT in GDR kochia (Fig. 4.9c) for *hot-DG* than *hot-D*, and similar difference was observed in GDS kochia at 24, 72, and 168 HAT (Fig. 4.9d). The translocation of dicamba to

ATL or BTL also confirmed these differences. For instance, when dicamba was mixed with glyphosate (*hot-DG*), less [^{14}C] dicamba was translocated to ATL at 168 HAT in GDR kochia (Fig. 4.9e), and at 24 and 168 HAT in GDS kochia (Fig. 4.9f). Also, less [^{14}C] dicamba translocation to BTL at 168 HAT in both GDR (Fig. 4.9g) and GDS kochia (Fig. 4.9h) was observed when dicamba and glyphosate were mixed. Furthermore, phosphor image analysis also supported that less [^{14}C] dicamba was translocated to shoots when dicamba was mixed with glyphosate than was applied alone in both GDR (Fig. 4.8d vs. 4.8c) and GDS (Fig. 4.8h vs. 4.8g) kochia.

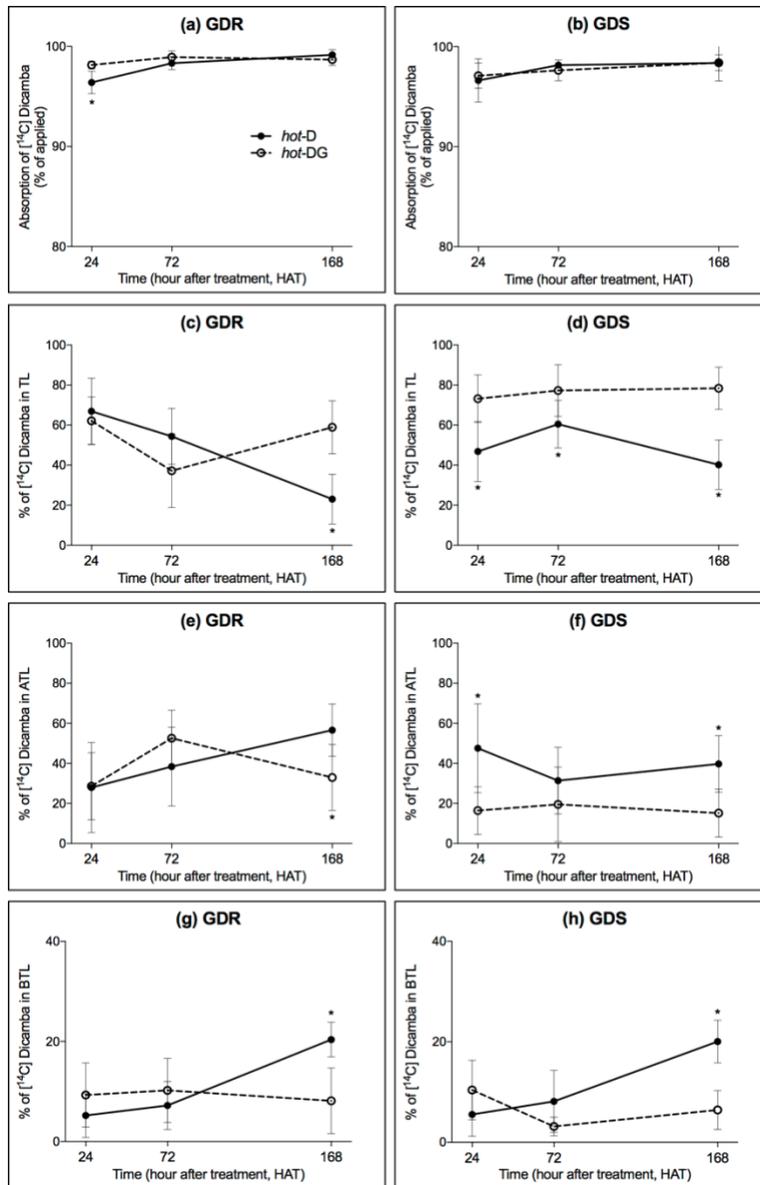


Figure 4.9 ^{14}C dicamba absorption (a and b), retained in treated leaves (TL, c and d), translocated to above treated leaf part (ATL, e and f) and below treated leaf part (BTL, g and h) in glyphosate- and dicamba-resistant (GDR, a, c, e, and g), and glyphosate- and dicamba-susceptible (GDS, b, d, f, and h) kochia when dicamba alone (*hot-D*, solid line), and glyphosate plus dicamba combination (*hot-DG*, broken line) was applied. (* P -value < 0.05, which indicate the levels of significance within each time point for different herbicide treatments; error bars represent standard deviation, n=8)

4.3.5 Discussion

The dose-response results confirmed that GDR kochia is resistant to both glyphosate and dicamba, whereas GDS kochia is susceptible to both glyphosate and dicamba. Furthermore, the GDR kochia exhibited low level of resistance to glyphosate, whereas, resistance to dicamba was high relative to the GDS kochia. Because of low level of resistance to glyphosate in GDR kochia, increased glyphosate dose provided better control of GDR kochia under both greenhouse and field conditions. In contrast increase in dicamba dose did not provide satisfactory control of GDR kochia in any conditions tested. Growers tend to increase the herbicide dose to achieve maximum weed control. However, our results suggest that increase in herbicide dose may not always provide good weed control, rather increase the selection pressure, which facilitates evolution of resistance. These practices are not sustainable and should not be recommended since they may drive weed populations to evolve a higher level of resistance (Godar et al., 2015; Jugulam et al., 2014).

Mixing herbicides with different sites/modes of action has been used widely to broaden the spectrum of weed control and delay the development of herbicide resistance (Beckie and Reboud, 2009; Johnson and Gibson, 2006). In this research both under greenhouse and field conditions, we found that combinations of glyphosate plus dicamba had antagonistic effect on GDR and GDS kochia control. When glyphosate was mixed with dicamba, the GDR kochia control was significantly decreased compared to the same dose of glyphosate applied by itself (Table 4.5 and 4.6). The GDS kochia was controlled using most of the herbicide combinations tested, primarily because high doses of glyphosate and/or dicamba can mask the antagonistic effect of reduced translocation of these herbicides in GDS kochia.

When applied in combination, the absorption of glyphosate and dicamba was enhanced at early hours than treated separately (Fig. 4.7 and Fig. 4.9). Especially glyphosate absorption was increased in both GDR and GDS kochia at 24 HAT (Fig. 4.7a and 4.7b); after that the difference in absorption was minimal, suggesting that mixing these two herbicides can accelerate absorption of both herbicides immediately after application. Accelerated absorption of dicamba possibly occurred because of the inclusion of adjuvant ammonium sulfate (Roskamp et al., 2013). The rapid absorption of ammonium ions can reduce the apoplastic pH (Husted and Schjoerring, 1995), which can enhance dissociation of the dicamba diglycolamine salt (in Clarity[®] formulation of dicamba) to form non-ionized dicamba acid and become more lipophilic. Once dicamba becomes more lipophilic it can be absorbed more quickly via waxy leaf cuticles, which are highly lipophilic (Hess and Foy, 2000; Sterling, 1994). However, this process could also increase the volatility of dicamba and upsurge the potential of dicamba drift due to presence of acid form of dicamba (Bauerle et al., 2015), yet not completely absorbed by the plant. On the other hand, glyphosate absorption could have been enhanced by the adjuvants included in Clarity[®] formulation, but additional study is needed to test this hypothesis.

Translocation of glyphosate was affected by dicamba regardless of time after application. When glyphosate was mixed with dicamba, less [¹⁴C] glyphosate was translocated and more was retained in treated leaves. This could occur as a result of rapid plant response to dicamba. As an auxinic herbicide, dicamba can cause rapid metabolic and physiological reactions within hours after application, which soon can lead to growth inhibition and reduction of transpiration and carbon assimilation (Grossmann, 2010). Glyphosate is mainly transported via phloem (Bromilow et al., 1993), which is highly dependent on the source-sink strength (Lemoine et al., 2013). Therefore, due to weakened source upon dicamba application, the translocation of glyphosate

may have been restricted compared to when glyphosate was applied alone. On the other side, reduced dicamba translocation was observed only at later time points when applied in combination. Glyphosate inhibits EPSPS enzyme and shuts down the shikimate pathway, which causes aromatic amino acid synthesis failure and stunts the growth of plants, and ultimately lead to plant death (Amrhein et al., 1980; De María et al., 2006). Within days, the glyphosate was translocated throughout the plant and shut down the shikimate pathway completely, soon after the carbon assimilation and phloem transport can cease. Therefore, the translocation of dicamba, which is also mainly facilitated by phloem (Bromilow et al., 1990; Chang and Vanden Born, 1968), would be significantly affected as a result of glyphosate-induced physiological alterations in plants.

In conclusion, though glyphosate plus dicamba combination is used to control a wide spectrum of monocot and dicot weeds in crops, this combination not necessarily is a good option to manage the stubborn weeds, such as kochia, in North America Great Plains. Our results clearly suggest that glyphosate plus dicamba combination has significant antagonistic effect on both GDR and GDS kochia, as a result of decreased translocation of these two herbicides resulting in reduced efficacy of both the herbicides. Therefore, if kochia is the major issue in the field, glyphosate plus dicamba combination should not be recommended, especially when glyphosate and/or dicamba-resistant kochia is present. Diversification of weed management tactics, such as inclusion of a third mode of action herbicide in the herbicide combination, or other non-chemical management practices such as tillage or cover crops are highly warranted to minimize the further development and spread of herbicide-resistant kochia.

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Appendix A - Abbreviations

Abbr.	Definition
ABA	abscisic acid
ABC	ATP-binding cassette
ABCB	ATP-binding cassette subfamily B
ACCase	acetyl CoA carboxylase
AFB	auxin-related F-box
AFLP	amplified fragment length polymorphism
ALS	acetolactate synthase
AMS	ammonium sulfate
ANOVA	analysis of variance
ARFs	auxin response factors
ATL	plant parts above treated leaf
ATP	adenosine triphosphate
BTL	plant parts below treated leaf
CA	California
CHS	chalcone synthase gene
CO	Colorado
CSUR	dicamba-resistant kochia from Colorado

CSUS	dicamba-susceptible kochia from Colorado
CT	Connecticut
D	dicamba
d/n	day/night
dNTP	deoxyribonucleotide triphosphate
DR	dicamba-resistant
DS	dicamba-susceptible
DTT	dithiothreitol
ED	effective dose
EDTA	ethylenediaminetetraacetic acid
EPSPS	5-enolpyruvate-shikimate-3-phosphate synthase
Fig.	Figure
G	glyphosate
GDR	glyphosate- and dicamba-resistant
GDS	glyphosate- and dicamba-susceptible
GIMP	GNU Image Manipulation Program
GR	growth reduction
GR	glyphosate-resistant
GST	glutathione- <i>S</i> -transferases

HAT	hours after treatments
hot-DG	[¹⁴ C] dicamba + glyphosate
hot-GD	[¹⁴ C] glyphosate + dicamba
HPLC	high performance liquid chromatography
HPPD	hydroxyphenylpyruvate dioxygenase
IAA	indole-3-acetic acid
ID	Idaho
KS	Kansas
KSUR	dicamba-resistant kochia from Kansas
KSUS	dicamba-susceptible kochia from Kansas
LSS	liquid scintillation spectrometry
MA	Massachusetts
MN	Montana
MOPS	3-(<i>N</i> -morpholino)propanesulfonic acid
ND	North Dakota
P1	Kansas kochia population 1
P2	Kansas kochia population 2
P450s	cytochrome P450 monooxygenases
PA	Pennsylvania

PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethane sulfonyl fluoride
POST	postemergence
PPO	protoporphyrinogen oxidase
PRE	preemergence
PS	photosynthesis
PVDF	polyvinylidene difluoride
PVPP	polyvinyl polypyrrolidone
qPCR	real-time polymerase chain reaction
RGB	Red, Green, and Blue
ROS	reactive oxygen species
SCF ^{TIR1/AFB}	Skp, Cullin, F-box containing complex
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorphisms
T1	17.5/7.5 °C
T2	25/15 °C
T3	32.5/22.5 °C.
TCA	trichloroacetic acid

TFA	trifluoroacetic acid
TIR1	transport inhibitor response 1
TL	treated leaf
TX	Texas
WAT	week(s) after treatment
WI	Wisconsin
WSSA	Weed Science Society of America