DISSERTATION

THE GENETICS AND PHYSIOLOGY OF A DICAMBA RESISTANCE TRAIT IN KOCHIA (*KOCHIA SCOPARIA* L. SCHRAD.)

Submitted by

David S. Belles

Department of Bioagricultural Sciences and Pest Management

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY DAVID S. BELLES ENTITLED **THE GENETICS, AND PHYSIOLOGY OF A DICAMBA RESISTANCE TRAIT IN KOCHIA (*KOCHIA SCOPARIA* L. SCHRAD)** BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Dr. Dale Shaner

Dr. Sarah Ward

Co-Adviser – Dr. Scott Nissen

Adviser – Dr. Phil Westra

Department Head – Dr. Tom Holtzer

Abstract of Dissertation

THE GENETICS AND PHYSIOLOGY OF A DICAMBA RESISTANCE TRAIT IN KOCHIA (*KOCHIA SCOPARIA* L. SHRAD)

Kochia is a problem in cultivated fields, waste lands and gardens throughout the western United States. Herbicides that mimic auxin are important for the control of kochia. In 1993, kochia was collected that did not respond as expected to dicamba an auxinic herbicide. In 1995, Montana and North Dakota reported auxinic herbicide resistant populations. From 1994 to 1998, 250 kochia samples from Colorado, Kansas, Montana, and Nebraska were tested for dicamba tolerance. Beginning in 1999, studies were conducted to investigate the genetics and physiology of the dicamba resistance trait identified in these populations and to evaluate resistance to fluroxypyr another auxinic herbicide used for kochia control. Four kochia accessions with different dicamba tolerance levels were evaluated under irrigated field conditions without competition for cross resistance to fluroxypyr. Five rates of dicamba and fluroxypyr were applied to the four kochia accessions. Dicamba control of dicamba resistant accessions was reduced. Fluroxypyr controlled all dicamba susceptible and resistant accessions tested. Auxinic herbicides have been shown to induce ethylene production in susceptible plants. The ethylene production of dicamba susceptible and resistant kochia when treated with dicamba and fluroxypyr was measured as a way to identify a resistant kochia plant and determine if it was cross resistant to fluroxypyr. The ethylene produced was measured over time and herbicide rate. Ethylene inhibitors and tobacco mutants were used to determine ethylene's contribution to observed herbicide symptoms of auxinic herbicides. Ethylene production by dicamba resistant kochia was lower over time and in lower

amounts as dicamba rate increased than susceptible kochia. Resistant and susceptible kochia responded similarly to fluroxypyr treatments. The ethylene inhibitor AOA did not reduce dicamba or fluroxypyr symptoms. Senescence was delayed in ethylene insensitive tobacco when treated with dicamba but not fluroxypyr. The inheritance of the dicamba resistant trait was studied. Resistant and susceptible inbred kochia lines were crossed and segregation patterns of eight F_2 families evaluated by treating them with 280 g ha $^{-1}$ dicamba. Fourteen days after treatment intermediate phenotypes were identified but resistant, intermediate, and susceptible plants did not segregate in a 1:2:1 ratio. The dicamba resistance trait in kochia is not due to a single nuclear gene and does not consistently fit simple modified ratios for a two gene system. At least two genes interact to produce the dicamba resistance phenotype observed.

David Stuart Belles
Philip H. Westra, Advisor
Bioagricultural Science and
Pest Management Department
Colorado State University
Fort Collins, CO 80523
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Chapter 1

Auxin, Herbicides, Kochia, and Resistance

David S. Belles*[1]

[1]*Department of Bioagricultural Sciences and Pest Management, Colorado StateUniversity, Fort Collins, CO 80523-1177*

*Corresponding author

Growth Regulator Herbicides.

Announced in 1944, 2,4-dichlorphenoxyacetic acid (2,4-D) and 2,4,5-trichloropenoxy acetic acid (2,4,5-T) were the first synthetic plant growth regulators recognized as selective herbicides. One of the most important and exciting characteristics of these compounds was their ability to kill plants at low concentrations. Doses of 100 ppm and 500 ppm were reported effective in killing field bindweed (*Convolvulus arvensis*). Generally, only 2.8 to 5.6 kg was needed per ha. Before these herbicides were discovered 45 t of table salt, 2.8 to 5.5 t of borax or 395 to 790 kg of sodium arsenite were required to treat one hectare (Hildebrand 1946). Besides the low concentrations required for effective control, 2,4-D was also highly selective, noncorrosive, nonirritating to the skin, non-explosive, and had no apparent adverse effects in feeding trials (Hildebrand 1946).

The discovery of the herbicidal activity of chlorophenoxyacetic acid compounds was made independently by four groups, two in the United States and two in the United Kingdom with work beginning between 1940 and 1943. The four groups were Templeton and associates at the Imperial Chemical Industries, U.K., Jones, American Chemical Paint Company, U.S.A., Nutman, Thornton, Quastel, Rothamsted Agricultural Experiment Station, U.K., and Kraus, Mitchell and associates, University of Chicago; Belstsville Experiment Station, U.S.A. (Troyer 2001). The first group to publish their findings was Kraus, Mitchell and associates with a report in Science, August, 1944 (Hamner and Tukey 1944)

Early in 1944, a project was started at Camp Detrick, MD to study the effect of more than 1100 synthetic plan growth regulating substances in corn germination tests.

Benzoic acid compounds were among those tested. The activity of the benzoic acids tested ranged from 57 to 33% of the activity of 2,4-D at a concentration of 10 ppm in water. When kidney bean seedlings were treated with 200 ppm of the compounds in water, one benzoic compound 3,5-dichloro-2-bromobenzoic acid expressed 144% of the activity of 2,4-D. The other benzoic compounds generally did not do as well (Thompson et al. 1946). Benzoic acid itself is physiologically inactive but adding a halogen, methyl, or nitro group activates the molecule. Substitutions on the 2-,3-, and 4- positions on the benzene ring have the most influence (Gilbert 1945).

Dicamba (3,6-dichloro-*o*-anisic acid, IUPAC, or 3,6-dichloro-2-methoxybenzioc acid) is a popular growth regulator herbicide in the benzoic acid chemical family. Dicamba may be used for the control of kochia in many monocot crops, including wheat and corn. In 2001, 26,000 kg of dicamba were applied to corn in Colorado alone (NASS database 2003).

Kochia Biology.

Kochia (*Kochia scoparia* L. Schrad), a member of the Chenopodiaceae family, is a common weed throughout the western United States infesting cultivated fields, waste lands, roadsides, and gardens up to 2591 m in elevation (Whitson 1996, Zimdahl 1998). Other common names for kochia include: Mexican fireweed, burning bush, summer cypress, and belvedere (Eberlein and Fore 1984). It is a native of southern and eastern Russia and part of the native flora of Afghanistan (Eberlein and Fore 1984, Holm et al. 1979). Kochia was introduced into the United States as an ornamental plant for backdrop plantings and hedges around gardens (Eberlein and Fore 1984).

Although kochia was rare in cultivated fields in North Dakota and Kansas in the 1920's, during the drought years of the 1930's it spread rapidly (Stevens 1965, Gates 1941). It was reported to spread again in Kansas, Colorado, and North Dakota during dry years in the 1950's and in 1976 (Becker 1978). It has now spread throughout the Great Plains and drier areas of the western United States. It is salt and drought tolerant and its spread may be related to soil moisture and reduced crop competition (Eberlein and Fore 1984). Kochia varies greatly in form and will grow from 15 cm to 1.83 m. In the presence of competition it can grow to 2 m tall. Without competition it is normally bushy and about 1 to 1.2 m tall (Eberlein and Fore 1984).

The entire plant has a cylindrical globose shape. Leaves are linear or lanceolate to narrow obovate, alternate and simple. They are 2 to 10 cm long, and 1 to 12 mm wide, turn red with maturity, and can be glabrous or glabrescent. Stems are erect or spreading with many branches and can be yellowish green, green, or have reddish stripes (Stubbendieck et al. 1997). Plants growing without competition with favorable moisture can have a root profile at maturity 7 m wide and over 2 m deep. In drought conditions roots can grow 5 m deep (Davis et al. 1967, Phillips and Launchbaugh 1958). Kochia germinates early in the spring and seedlings are tolerant to frost (Stevens 1965). The optimum temperature for germination is 16 C but germination was 76% or better at temperatures ranging from 4 to 41 C (Larcher et al. 1963). Germination was 60% or greater in soil at pH of 2 to 8 but over 8, germination declined rapidly. Seeds were able to germinate and establish in salt conditions as high as 10,000 ppm and under moisture stress of 13.2 bars (Evetts and Burnside 1972).

Kochia flowers from 57 to 100 days after emergence, responding to short days (13 to 15 h) to initiate flowering (Bell et al. 1972). The flowers are inconspicuous in the upper leaf axils on the plant and contain 5 sessile stamens per flower. They can be perfect or unisexual and apetalous but are subtended by pubescent leafy bracts. Together they form short, dense, bracted spikes. Some plants are floriferous for most of their height. Fruits are utricles, depressed and globose. They are enclosed in a five winged persistent pericarp (Stubbendick et al. 1997).

Although each flower produces only one seed, kochia is a prolific seed producer. Over 14,000 seeds are typically produced per plant (Thill et al. 1993). Seeds have limited longevity in the soil, however, and germination declined from 40 to 5% after 1 year of burial to nearly 0% after 2 years (Burnside 1981). Seeds are dispersed as the plant rolls or tumbles across the ground blown by the wind. As the plant matures the stem slowly looses flexibility. Then andstrong winds can break the plant at the ground surface allowing the plant to roll and disperse its seeds (Eberline and Fore 1984).

Kochia is a problem in the late harvested crops, soybean and sugar beets, and in small grains and flax (Bell et al. 1972). Early germination may result in large plants at the time of herbicide application that reduce control (Eberline and Fore, 1984). Kochia also causes hay fever and other respiratory problems in some humans and may cause nitrate poisoning, bloat, and photosensitization in livestock. The forage value can be good for livestock and wildlife while it is young but palatability decreases with maturity (Stubbendick et al. 1997).

Herbicide Resistance.

The spread of kochia in the United States, since its introduction, has followed a sigmoidal curve, with the greatest expansion in the 1950's and 60's. Its range now covers almost the entire country. Kochia is very competitive and may even have some allelopathic abilities. Kochia was one of the first weeds to develop resistance to the sulfonylurea and triazine herbicides (Primiani et al. 1990). Populations resistant to sulfonylurea herbicides are also resistant to imidazolinone herbicides as these herbicides affect the same target site in the plant, acetolactate synthase (ALS) or acetohydroxy acid synthase (AHAS). Resistant populations were selected for by repeated use of these chemistries for control of kochia or other weeds in the cropping system. The resistant populations have developed independently in different areas through selection over a wide geographical area. There are at least six mutations of the ALS gene which confer resistance to ALS or AHS inhibitors in kochia (Shaner 1991).

Greenhouse experiments with resistant and susceptible kochia have revealed populations that are 2 to 400 times more resistant to sulfonylurea herbicides than susceptible populations as measured by shoot dry matter accumulation (Saari et al. 1990). Resistant kochia plants were 2 to 180 times more resistant to five sulfonylurea herbicides and one imidazolinone herbicide than susceptible plants as measured by shoot biomass reduction in the greenhouse (Friesen et al. 1993).

Sulfonylurea resistant and susceptible biotypes have nearly equal relative competitive abilities. The average relative competitive ability was .75 and .85 for resistant and susceptible kochia respectively. There was no difference in biomass and leaf area. However, resistant biotypes did germinate faster than susceptible biotypes, and at cooler temperatures. At 8 C, the maximum germination for resistant kochia was 192

hours and 336 hours for susceptible kochia. At 18 C the maximum germination for resistant and susceptible kochia was 110 and 144 hours, respectively. Both resistant and susceptible biotypes germinated in the same amount of time at 28 C (Thill et al. 1993). Therefore, there would be no selection pressure to return to a susceptible population in the absence of triazine herbicide pressure.

Resistance to auxinic herbicides was first documented in 1957 where one population of spreading dayflower (*Commelina diffusa* Burm.) in sugarcane fields in Hawaii was reported to be five times more resistant than susceptible populations and in Canada where wild carrot (*Daucus carota* L.) was treated regularly along highways. (Heap 1997). Other resistant species have been found in Canada, Spain, France, New Zealand, Indonesia, Malaysia, and the Philippians (Heap 1997).

Variability in kochia control by auxinic herbicides was observed more than 30 years ago (Bell et al. 1972b). The most tolerant kochia populations to 2,4-D required twice as much herbicide to obtain the same level of control as the most susceptible. 2,4-D at 350 g ha^{-1} had a greater effect on the most susceptible accession than 700 g ha^{-1} on the most tolerant. In the same way, it required almost twice as much dicamba to injure the most dicamba tolerant accession as the most susceptible. Dicamba at 280 g ha^{-1} had as great an effect on one accession as 560 g ha^{-1} had on the most tolerant accession. Interestingly, the response of kochia selections to dicamba was independent of the response to 2,4-D, offering no indication of cross resistance between the herbicides even though their symptomology was very similar. With this degree of variation in the population, selection for more resistant populations with repeated use would be expected (Bell et al. 1972b).

In 1994, kochia populations not controlled by field rates of dicamba were recognized. Susceptible plants were severely injured with 35 g ha^{-1} dicamba while resistant plants required 210 g ha^{-1}. ED_{50} values were 31 and 143 g ha^{-1}, for susceptible and resistant plants, respectively. Resistant biotypes were isolated with an R/S ED_{50} ratio of 4.6. The resistance was not due to reduced absorption or altered translocation. An increase in metabolism in resistant plants was detected verses susceptible plants. However, it was noted that symptoms appeared in the susceptible plants before metabolism was detected in the resistant plants and only small amounts of metabolites were found in the shoot apical meristem which did not show symptoms. Reports of reduced dicamba control continued in following years but the resistance problem did not develop as quickly as the sulfonylurea and triazine resistance problems. Therefore, resistance was assumed to be a quantitative trait, one not conferred by a single dominant or partially dominant gene, but many genes acting together (Cranston et al. 2001).

Dicamba tolerance has been extensively studied in wild mustard (*Sinapis arvensis* L.) and yellow starthistle (*Centaurea solstitialis* L). The 23 fold lower R/S ratio for dicamba tolerance in kochia than in wild mustard may indicate a different genetic basis of resistance (Cranston et al. 2001). However, this is not confirmed and there may be some important similarities. Similar to kochia seed, germination of the resistant biotype of wild mustard at 24, 30 and 35 C was higher than the susceptible biotype. The resistant biotype was also more tolerant to cold temperature (7 C). Morphologically the resistant wild mustard biotype was shorter, more branched, and had a smaller root system. Its leaves were smaller, darker green and had more chlorophyll. Concentrations of cytokinins were 4 times greater as well. Cytokinin promotes lateral bud formation as

well as to reduce shoot and root growth. This is consistent with the observation that the resistant plants were shorter and more branched. Cytokinins also promote chloroplast development and chlorophyll synthesis (Hall and Romano 1995). Senescence was also delayed in the resistant wild mustard. The multiple morphological changes might suggest a quantitative resistance trait; however, a singe gene mutation in *A. thaliana* conferred auxin resistance with pleiotropic morphological effects (Estelle and Somerville, 1987).

Auxinic herbicide resistant yellow starthistle was first observed in 1998 in Washington. The level of resistance to picloram was 3 to 35 times depending on the experiment and growth conditions. Cross resistance was observed to clopyralid, dicamba, and fluroxypyr but not to triclopyr or 2,4-D. Metabolism was not different between resistant and susceptible biotypes but absorption and acropetal translocation were reduced. Picloram also induced ethylene production. Susceptible plants produced 20 times more ethylene than resistant plants. It was proposed that the affinity of an auxin binding protein on the plasma membrane for picloram was reduced in the resistant biotype. This could explain the reduction in absorption, acropetal translocation, and ethylene production observed (Fuerst et al. 1996); however, there was no difference between resistant and susceptible biotypes in the absorption or translocation of clopyralid. Metabolism was greater in the resistant biotype after 96 h but symptoms developed within 5 h in susceptible plants. Therefore, the increased metabolism is not the cause of resistance. Ethylene production was only induced in the susceptible biotype when sprayed with clopyralid. This result is the same as when the plants were treated with picloram. When exposed to ethylene the wild mustard became epinastic but yellow starthistle did not. Therefore, it was concluded that ethylene was not involved in the

action of clopyralid in yellow starthistle and resistance must be due to differences at the target site such as signal transduction or an auxin receptor (Hall et al. 1993, Valenzuela-Valenzuela et al. 2001).

The role of ethylene in auxinic herbicide action was further investigated in yellow starthistle using the ethylene inhibitors (E)-L-2-[2-(2-amino ethoxy)vinyl]glycine (AVG), silver thiosulfate (STS), and aminooxy acetic acid (AOA). Although ethylene production was prevented, epinasty, necrosis, and growth inhibition were not prevented. It may be that a characteristic of yellow starthistle is its insensitivity to ethylene. Fumigating with ethylene gas for 48 h did not cause auxinic herbicide symptoms to develop (Valenzuela-Valenzuela et al. 2002). Other plants such as tomato, wild mustard, rapeseed, and sunflower are more sensitive (Hall et al. 1993, Grossmann and Schmulling 1995, and Hall et al. 1985).

In wild mustard the production of 1-aminocyclopropane-1-carboxylic acid (ACC) and 1-malonyl aminocyclopropane-1-carboxylic acid (MACC), precursors to ethylene, and ACC synthase was quantified after application of picloram at 100 g ha^{-1}. After pretreatment with aminooxyacetic acid (AOA) and ACC was supplied, tissues of both resistant and susceptible plants produced ethylene, proving ACC synthase was still functional (Hall et al. 1993).

In another study, Deshpande and Hall (1995) found that flash-induced light scattering signals from resistant and susceptible wild mustard protoplast indicated there were ATP-dependent activity differences between biotypes. The signal from resistant protoplasts was not effected by 50 μM picloram where as the amplitude of the signal decreased 40% in the susceptible protoplasts in 20μM and was reduced to 0 mV in 50μM

picloram. Adding calcium channel blockers reduced the signal and picloram activity was inhibited by adding a calcium ionophore. Therefore, since picloram affects proton and Ca^{++} flux of the susceptible biotype only, calcium may be significant in regulating picloram resistance (Deshpande and Hall, 1995).

The ability of picloram to inhibit indole acetic acid (IAA) binding in resistant biotypes was also reduced. The concentration required to inhibit 50% IAA binding in resistant and susceptible auxin binding protein (ABP) preparations was 104 ± 2.6 μM and 92 ± 4.5 nM, respectively. There was no difference in the concentration required for 2-(2-Methyl-4-chlorophenoxy)propionic acid (MCPP) or 4-chloro-2-methylphenoxy)acetic acid (MCPA). There was a difference for dicamba, although not as large. These observations agreed with the inhibition of seedling growth. There was no inhibition of seedling growth by MCPP while there was inhibition by picloram and dicamba. However, MCPA did inhibit growth differently in resistant and susceptible seedlings although inhibition of ABP binding was the same (Webb and Hall 1995).

Picloram also increased hydrogen ion efflux in a susceptible biotype but did not affect H^+ efflux in the resistant biotype. MCPP increased H^+ efflux in both biotypes and adversely affected activity on both resistant and susceptible biotypes. After pre-incubation the response of the resistant biotype was modulated by MCPP but not by the other herbicides. This may indicate conformational changes in an IAA-binding site that occurred after binding with IAA, altering the response to the herbicide. This effect was not observed in susceptible biotypes. Therefore, it was proposed that there may be two distinct binding sites in susceptible and resistant biotypes with high and low affinity sites.

The resistant biotype may lack the high affinity site that may be affected more by the auxinic herbicides (Webb and Hall 1995).

Right-angle light scattering showed volume changes in protoplasts of wild mustard. When resistant and susceptible protoplasts were treated with IAA both initially shrank and then swelled. However, when treated with picloram only swelling in susceptible protoplasts was observed and there was no shrinkage. Both swelling and shrinking occurred again in the resistant protoplasts. Incubation with a calcium channel blocker reduced the initial shrinkage. It was hypothesized that for the picloram to be active, a disruption of calcium transport must occur. In resistant biotypes the disruption of calcium transport was reduced (Deshpande and Hall 1996, and Deshpande and Hall 2000).

Auxinic herbicide resistant kochia and bedstraw (*Galium aparine* L.) have been identified. Bedstraw tolerant to fluroxypyr may have another mode of action. There was no difference in absorption between resistant and susceptible bedstraw and differences in translocation did not explain the difference in tolerance. Both resistant and susceptible biotypes showed some symptoms during the 7 days following treatment. This may suggest detoxification as the mechanism of resistance to fluroxypyr in bedstraw (Hill et al. 1996).

Abscisic acid (ABA) was also implicated in auxin herbicide action. ABA is produced by oxidative cleavage of the polyene chain of a C_{40} epoxyanthophyll precursor, formed from violaxanthin in carotenoid biosyntheis. ABA is involved in regulating stomatal closure and subsequent reduction in transpiration and CO_2 assimilation. When plant roots were treated with 10 μM quinmerac, ethylene synthesis in shoot tissues

increased as well as xanthoxal and ABA levels. In addition, stomatal aperture, CO_2 assimilation, and chlorophyll content decreased, while H_2O_2 levels and DNase activity increased (Grossmann et al. 2001). ABA levels increased 24 times over 24 h following treatment with IAA. 1-amino-cyclopropane-1-carboxylic acid (ACC) synthase and ethylene production also increased. ACC production peaked after 3 h and ethylene production peaked after 5 h (Grossmann et al. 2001).

Similar results were observed when the plants were treated with quinmerac, α-naphthalene acetic acid (NAA), dicamba, and picloram. ABA accumulation and plant growth was inhibited by amino-ethoxyvinyl-glycine (AVG) and cobalt ions, ethylene inhibitors. Treatment with ethephon stimulated ABA production and reduced shoot growth. Never ripe tomato that is ethylene insensitive was treated with IAA, quinmerac, NAA, picloram, and dicamba. After 24 h the accumulation of xanthoxal had been completely eliminated. The accumulation of ABA was also greatly reduced but not eliminated. Auxin may stimulate ethylene production which results in ABA accumulation by triggering new synthesis of ABA. Following quinmerac treatment the levels of xanthoxal and ABA increased but not of neoxanthin, violaxanthin, antheraxanthin, or β–carotene. It was concluded that the main target site of auxin induced ethylene was the oxidative cleavage of 9'-cis'xanthophylls in ABA biosynthesis (Hansen and Grossmann, 2000).

Auxin Physiology and Genetics.

Auxin, an important plant hormone with many effects on plants, is synthesized from tryptophan and in a tryptophan independent pathway in which indole-3-acetonitrile

is an intermediate (Leyser 1997, Normanly 1997, Zazimalova and Napier 2003). Auxin is produced in the apical meristem and moves in a polar manner from the shoot meristem to the base of the stem. This results in an auxin gradient in the plant. In the root, auxin moves acropetally, through the central cylinder and basipetally through the outer root layers. Basipetal movement has been linked to root gravitropism. Auxin can also move laterally across shoots and roots, stimulated by a change in the gravity vector (Muday 2001).

This lateral auxin transport was hypothesized by Cholodny and Went to drive the differential gravitropic response. Genetic evidence supports this hypothesis. Gene expression occurs in gravitropic stimulated plants and gene expression is asymmetric in stimulated roots and shoots. Auxin transport inhibitors block gene expression and gravitropic bending. Plants growing without gravity fail to respond to a horizontal orientation. There are some inconsistencies with the Cholodny-Went hypothesis; however, roots over respond to gravity moving past vertical when orientating from horizontal. Also, roots still respond to gravity when grown in high auxin concentrations (Muday 2001).

IAA can move into a cell passively as it is hydrophobic when protonated. Once inside, the molecules are deprotonated, becoming hydrophilic, preventing them from moving back across the cell membrane. A second way IAA enters a cell is through an influx carrier. This carrier is believed to be encoded by the AUX1 gene or a homolog. IAA moves out of cells through an efflux carrier. It is this auxin efflux carrier that regulates the amount and direction of auxin transport. Arabidopsis mutants with putative auxin influx, aux1 or efflux carriers, agr1, eir1, pin2, or wav6 have agravitropic roots.

Other mutants with impaired growth inhibition by auxin such as aux, axr1, axr4, axr6, and dwf also have altered root gravitropism (Muday 2001).

Auxin is also needed for phototropism. Phototropic mutants nph4, msg1, and tir5 show loss of phototropism in hypocotyls, loss of auxin induced growth curvature, insensitivity to auxin transport inhibitors, and a reduction in phototropic and gravitropic bending, apical hook maintenance, and sensitivity to growth regulation and gene expression by auxin. NPH4 encodes auxin response factor 7 (ARF7). These auxin response factors are needed for phototropism. The actual bending in tropic responses is due to differential cell elongation caused by pH changes in the cytoplasm and apoplast, modification of potassium channels, and cell wall loosening enzymes (Muday 2001).

By affecting K^+ channels auxin affects cell elongation. Both auxin and extracellular K^+ ions are necessary for growth. Auxin induced growth with 1-10 mM KCl after 20 min. There was no growth without K^+ ions or auxin. Therefore growth and proton secretion depend on extracellular K^+ ions and the absorption of K^+ mediated by K^+ channels in the plasma membrane (Claussen et al. 1997).

Auxin is perceived in the plant by auxin binding proteins (ABP). ABP1 is an auxin binding protein with two 22 kDa subunits. It was shown to be associated with docking proteins on the plasma membrane where it is present in low concentrations. It is also found in high concentrations in the endoplasmic reticulum (ER). Auxin is present inside and outside of cells and could be perceived in either location. It might be a member of the G-protien coupled receptor (GPCR) family. A signal is initiated when auxin binds to the ABP1-docking protein complex. Several pathways are possible after

that. Phospholipase A_2 (PLA_2) can be activated leading to the production of lipid secondary messengers that activate H^+-ATPases by phosphorylation (MacDonald 1997).

Several auxin mutants have been identified in *Arabidopsis*. They demonstrate auxin's many effects on plants. Two of the most important are the aux and axr mutants. Aux1 is an auxin uptake mutant. Its effect is to reduce the intracellular auxin concentration compared to the wild type. One phenotype is rapid root growth. Auxin is normally inhibitory to growth in the root tip. AXR1 is involved in the early stage of auxin signal transduction. Phenotypically, Axr1 mutants have short highly branched shoots, and long highly branched roots with a slowed gravitropic response (Leyser 1997). AXR1 encodes a protein with an amino acid sequence with homology to the N-terminus of E1-ubiquitin-acting enzyme. The enzyme involved in the first step in the ubiquitin pathway AXR1 encodes a component of the E1 enzyme needed to catalyze activation of RUB1. (Møller and Chua 1999).

Axr4 mutant roots respond slowly to gravity and produce a reduced number of lateral roots whose elongation is also auxin resistant. There is little effect on the shoot. Axr2 mutants have a cell elongation defect. Their roots are auxin resistant, agravitropic, and have reduced numbers of root hairs. Their shoots are dwarfed with crinkled leaves and bolting stems are agravitropic. Axr3 mutants over-respond to auxin and have highly branched agravitropic roots, curled epinastic leaves and strong apical dominance. The roots are auxin resistant (Leyser 1997).

Ethylene and Auxin in Programmed Cell Death.

An important interaction of auxin and ethylene in plants is programmed cell death or apoptosis. Programmed cell death is carefully studied in animals because of its

importance in cancer physiology. Cancerous cells loose their apoptotic characteristics and continue to live after they would normally die. Many of the processes in animal programmed cell death are similar to those in plants (de Jong et al. 2002, Chae and Lee 2001). Apoptosis is a genetically defined process, and is part of the normal life cycle of multicelled organisms (de Jong et al. 2002). Apoptosis is different from necrosis, another process in which cells die. In necrosis, the plasma membrane ruptures but the event is not genetically programmed and is instead caused by some external factor. Apoptosis is physiologically characterized by biochemical and cellular processes including membrane blebbing, condensation of the cytoplasm and in animals, internucleosomal cleavage of DNA (Chae and Lee 2001).

In plants, auxin and ethylene interact to regulate programmed cell death. Tobacco hybrids expressing temperature apoptotic lethality symptoms had high auxin levels. Vigorously growing plants without lethal symptoms did not have increased IAA levels. The lethal symptoms were suppressed by the addition of 2,3,5 triiodobenzoic acid (TIBA), an auxin transport inhibitor. Auxin induces ethylene production which in turn induces apoptosis (Yamada et al. 2001). Aminooxy acetic acid (AOA), an inhibitor of pyridoxal enzymes and of ethylene production through the inhibition of ACC production, the ethylene precursor, also suppresses DNA fragmentation and lethal symptoms in tobacco and tomato (Ables 1992, de Jong et al. 2002, Yamada et al. 2001). Furthermore, ethylene treatment induced senescence and DNA breakdown in tomato and the regulation of DNA fragmentation during carpel senescence in *Pisium sativum* (de Jong 2002, Orzaez and Granell 1997).

Ethylene is unable to induce cell death alone. Cell death induced by campothecin, a topo isomerase inhibitor and a known inducer of apoptosis in animal cells, was reduced but not eliminated by AVG and STS, two ethylene inhibitors. The addition of ethylene eliminated the inhibition by the inhibitors and increased cell death over campothecin alone (de Jong et al. 2002). Calcium influx with ethylene is sufficient to induce programmed cell death (de Jong et al 2002). Ethylene also up-regulates a gene encoding a calmodulin binding protein involved in plant senescence and is evidence for Ca^{+2}/CAM mediated signaling in ethylene action (Yang and Poovaiah 2000).

Reactive oxygen species (ROS) are also involved in ethylene regulated programmed cell death. Ethylene regulates the release of ROS during campothecin induced programmed cell death (de Jong et al. 2002). Under carbon starvation, ethylene increased ethylene production, activation of NADPH oxidase or other ROS generating enzymes, leading to superoxide production and cell death (Chae and Lee 2001). Hydrogen peroxide is also produced following ethylene treatment in conjunction with calcium. No oxidative burst was observed in the presence of AVG or STS, indicating the importance of ethylene in H_2O_2 and ROS production; although, ethylene alone cannot produce H_2O_2 or ROS (de Jong et al. 2002).

Ethylene causes cell death at particular phases of the cell cycle. The most mortality occurs at the G_2/M phase with a second rise in mortality beginning in G_1 and peaking in the S phase. Ethylene induced mortality was characterized by nuclear shrinkage and DNA fragmentation at G_2/M and delayed the entry of viable cells into mitosis by 1 h. This may be a lag time necessary for DNA to recover from damage;

however, 1/3 of the cells didn't pass the DNA checkpoint and exited the cell cycle, entering the death process (Herbert et al. 2001).

Ethylene Physiology and Genetics.

Ethylene is produced from methionine that is first converted to S-adenosyl-L-methionine (SAM). S-adenosyl-L-methionine is then converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. ACC oxidase then catalyzes the conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene. The production of ethylene is under both positive and negative feedback control (Kim et al. 2001). The pathway is regulated by changing the level of ACC synthase activity through increased transcription for the enzyme or a post-transcriptional regulation mechanism. The conversion of SAM to ACC synthase is the rate limiting step (Yang and Hoffman 1984). The IAA concentration is correlated with the ACC concentration and ethylene production rate. Auxin stimulates ethylene production by inducing ACC synthase in mungbean (*Vigna radiate* L. Wilczek) and pea seedlings (*Pisum sativum* L.) (Yoshii and Imaseki 1981, Yoshii and Imaseki 1982, and Peck and Kende 1995). External stresses such as wounding, pathogen invasion, and flooding can induce ethylene biosynthesis as well (Abeles 1992). Ethylene has many physiological effects on a plant including: germination, apical hook formation, seedling emergence, shoot growth, apical dominance, leaf emergence and expansion, stem elongation, root elongation, gravitropism, and root formation as reviewed in (Smalle and Van Der Straeten 1997). Seeds with the etr1 mutation that do not sense ethylene, had poor germination compared to the wild type. Germination was enhanced with the addition of giberellic acid (Bleeker

et al. 1988). Ethylene may alter the promoter inhibitor ratio of GA and ABA to promote germination (Esashi 1991).

Apical hook curvature was enhanced by ethylene treatment (Guzmán and Ecker 1990, Kieber et al. 1993). HLS1 mutants that encode ethylene inducible N-acetyltransferase over-expression have constitutive hook curvature. The gene controls differential growth by regulation auxin activity and or transport (Lehman et al. 1996). The apical hook formation is due to cross talk between ethylene and auxin pathways (Smalle and Van Der Straeten 1997).

Ethylene induced three responses that allow seeds to push through hard soil. Treatment with ethylene results in as short thick hypocotyls and roots plus an exaggerated apical hook, this is the triple response. A physical impedance boosts ethylene biosynthesis as well (Goesch et al. 1996).

The apical hook opening was shown to be due to a reduction in ethylene biosynthesis or sensitivity (Goeschl et al. 1967). A constitutive ethylene response mutant ctr1 showed delayed de-etiolation (Kieber and Ecker 1993, Kieber et al. 1993). Ethylene reduced hypocotyl, root cell elongation and increased radial expansion of root cells (Osborne 1982). It caused the corticle microtubules to reorient and switch from elongation to radial expansion (Shibaoka 1994) and it may regulate cell wall peroxidases that control cell wall extension and cell growth (Ridge and Osborne 1971). Control of apical dominance is generally attributed to auxin and to cytokinin ratios; however, there still may be an auxin ethylene interaction due to sensitivity and transport (Romano et al. 1993, Smalle and Van Der Straten 1997). The hls1 mutant has reduced apical

dominance. The HLS1 gene intersects the auxin and ethylene pathways (Lehman et al. 1996).

Leaf emergence is associated with a peak in ethylene production and accelerates leaf emergence (Ievinsh and Kreiebergs 1992). The expansion of the leaf is regulated by ethylene by suppressing cell enlargement but not division (Kieber et al. 1993, Rodrigues Pousada et al. 1993). The ctr1 mutant has a reduced stature and unexpanded leaves. Ethylene insensitive mutants usually have larger rosettes than wild types due to greater cell enlargement (Ecker 1995, Hua et al 1995).

Epinastic petioles are characterized by cells on the adaxial side that elongate faster than cells on the abaxial side. Auxin causes epinasty in transgenic plants with reduced ethylene biosynthesis or sensitivity levels and is evidence for an ethylene independent response pathyway (Romano et al. 1993). However, there may be a direct ethylene response pathyway since transgenic tomato plants overproducing ACC synthase have epinastic petioles (Zobel 1973, Kelley and Bradford 1986, Ursin and Bradford 1989).

Ethylene inhibits stem elongation. An increase in ethylene modulates the ABA-GA ratio resulting in stem elongation (Hoffmann-Benning and Kende 1992). Ethylene inhibitors decrease stem curvature in shoot gravitropism (Philosoph-Hades et al. 1996). Ethylene also affects flowering time (Kieber et al 1993). Mutants constitutively producing ethylene have a late flowering phenotype (Smalle and Van Der Straeten 1997).

Exogenous ethylene decreased root elongation and increased radial expansion of root cells (Jackson 1991, Abeles et al. 1992). Roots of axr1, axr2, axr3 and aux1 mutants are insensitive to auxin and ethylene (Lee et al 1990). Ethylene appears to play a role in

regulating the helical movement of the root cap (Woods et al 1984). Reduced ethylene action weakened the roots ability to penetrate compact media (Zacarias and Reid 1992). Mutant dgt tomato roots show no lateral branching normally promoted by ethylene and have decreased sensitivity to auxin and ethylene (Zobel 1972, Kressin Mudah et al. 1995). However, in many species ethylene has been shown to promote the growth of root hairs (Abeles et al. 1992).

Ethylene perception is controlled by a family of five integral membrane receptors, ETR1, ETR2, EIN4, ERS1, and ERS2. The receptors are similar to the 2 component histidine kinase regulators found in bacteria and fungi. A functional ethylene signaling pathway is required for resistance against some pathogens (Stepanova and Ecker 2000). Ethylene is involved in the regulation of genes involved in plant defense and the senescence of carnation flower petals and fruit ripening (Deikman 1997). Mutations in the Arabidopsis gene AXR1 are characterized by auxin insensitivity and reduced sensitivity to ethylene in seedling roots and apical hooks (Stepanova and Ecker 2000). A single mutation in any one of the five genes is sufficient to confer ethylene insensitivity throughout the plant. Ethylene is a negative regulator of the ETR1 receptor family (Hua and Meyerozitz 1998). Dominant insensitivity could result from mutations that block ethylene binding (Hall et al. 1999).

Herbicide Resistance Population Genetics.

Several factors influence development of herbicide resistant weed populations: genetic variation, initial mutation frequency, selection intensity, generation time, mode of inheritance, gene flow, and relative ecological fitness (Warwick 1991). Before an

herbicide resistant trait can be selected there must be genetic variation in that resistance trait for selection to act upon. Adaptation to herbicide selection pressure is possible only if resistance genes are present in the population. A resistance trait with a large phenotypic effect will allow the survival of a few individuals in a single generation (Jasienuik et al. 1996). If these individuals have good fitness they will produce seed and the herbicide resistant population development will be relatively rapid.

Mutation is the major source of genetic variation in a population where herbicide resistance has not been detected before. In any population new mutations are continually occurring andsome of these may confer herbicide resistance. The typical rate of spontaneous mutation used in herbicide resistant population models is 10^{-5} to 10^{-6} gametes per locus per generation for a single, nuclear gene in models of herbicide resistance evolution. The actual rates of mutation to herbicide resistance, however, are unknown for weed species (Jasienuik et al. 1996). A high initial frequency of resistance genes will result in the rapid establishment of an herbicide resistant population once herbicides are used (Preston and Powles 2002). Rigid ryegrass, (*Lolium rigidum*) resistance to ALS herbicides is about 100 times more frequent than expected (Preston and Powles 2002). In reality the initial frequency of resistance alleles in a population are lacking and hard to obtain (Jasienuik et al. 1996). The frequency of herbicide resistant individuals in any population is assumed to be about 10^{-6} (Maxwell and Mortimer 1994). The initial frequency of resistant plants in a population may be estimated using mutation selection equilibrium theory. The frequency of a dominant resistant plant with little fitness cost is 1.09×10^{-4}. This is higher than expected and may explain the rapid selection of ALS resistant populations (Jasienuik et al. 1996).

If herbicide resistance is due to a single dominant resistance allele of 1 X 10^{-6} mutation rate, at least 1 resistant plant would be expected in a weed population of 5 plants m^{-2} in an area of 30 ha. If the mutation rate was 1 X 10^{-8} you would need 50 plants m^{-2} in the same area (Jasienuik et al. 1996). However, variability is not the same in all species (Tranel and Wright 2002).

Within a population, variation is higher when unexposed to an herbicide. A strong herbicide selection pressure will limit variation of the trait that selection is acting on and the mortality of most of the individuals in the population due to the herbicide will create a genetic bottleneck. Genetic bottlenecks reduce genetic variation in the population if the population size is reduced dramatically as would be the case when an herbicide is applied (Freeman and Herron 1998).

A population's response to selection depends on the population structure. The more heterogeneous a population, the more likely it can adapt to herbicide selection pressure (Warwick 1991). Herbicides are highly effective and therefore exert a strong selection pressure on weed populations. Selection is the most important factor determining the increase in resistant individuals in a population. The greater the selection pressure against susceptible seedlings the faster the rate of selection and spread of resistant plants. Herbicides with strong selection potential usually affect single target sites and a specific mechanism of action, have long soil residuals, and are used year after year (Jasienuik et al. 1996).

Selection intensity is determined by the specific mode of action, the activity of the herbicide, soil residual, and application frequency (Warwick 1991). Herbicides with a long soil residual are more likely to develop resistance because they exert selection

pressure over a longer period of time. Post emergence herbicides applied late also control later flushes and may also exert a strong selection pressure (Morrison and Devine 1994). The longer a species seeds survive in the soil the slower resistance will increase because susceptible plants will germinate and contribute susceptible alleles to the population. The number of herbicide resistant individuals always increases when an herbicide is used; however, it may take many years for the frequency of resistance weeds to reach a noticeable level (Gressel 1986). Herbicide rotation is more effective in slowing the spread of resistance a resistant population when the resistant mutant is less fit than the wild type (Jasienuik et al. 1996).

The mode of inheritance also affects how quickly an herbicide resistant population will be selected. A rare favored dominant allele will spread faster than a recessive one. Heterozygotes will express at least part of the phenotype (Jasienuik et al. 1996). Polymorphism is preserved as the heterozygotes survive leading to quick spread and establishment of resistant plants (Darmency 1996). ALS resistance is conferred by a single dominant nuclear encoded gene and might account for the high frequency of occurrence of resistance to ALS inhibitors relative to some other herbicides (Tranel and Wright 2002). The frequency of recessive alleles are two to three orders of magnitude greater than dominant resistant alleles; however, herbicide resistance is mostly a dominant trait because herbicides are highly target specific and interfere with a single enzyme in major metabolic pathways. Repeated applications of highly effective herbicides exert a strong selection pressure (Jasienuik et al. 1996).

A self pollinated species has higher probability of containing a recessive herbicide resistance mutation. Natural selection strongly favors non-recessive mutations in random

mating or predominantly outcrossing species (Jasienuik et al. 1996). By rapidly increasing the frequency of recessive homozygotes, selfing reduces the chance of loosing a newly arisen recessive mutation (Jasienuik et al. 1996). A recessive herbicide resistant allele was selected for in green foxtail a highly self fertilized species (Darmency 1994).

Polygenic inheritance is rare. Gene exchange is necessary to combine favorable alleles for herbicide resistance and would require a large population of an outbreeding species to ensure the presence of a large number of partial resistance genes (Darmency 1996). If these conditions exist recurrent selection may result in a progressively resistant population from generation to generation with changes in gene frequency at several loci (Gasquez 1997). In addition application doses have to be low otherwise the plant would not be resistant enough to survive the herbicide application (Gasquez 1997).

Quantitative inheritance for herbicide resistance generally has not been observed because resistance genes are diluted by a susceptible genetic background. The soil seed bank has a buffering effect, additive polygenic mechanisms are more prevalent in self compatible or allogamous species, and quantitative resistance mechanisms only lead to a two to three times increase in resistance (Gasquez 1997). Phenoxy herbicides have a lower selection pressure than other types of herbicides. They have a shorter soil residual time and generally lower effectiveness. This allows some susceptible plants to escape (Gressel 1986). The lower selection pressure also allows resistance alleles to survive and recombine perhaps enabling a quantitative resistance trait to be selected.

Cytoplasmic inheritance of an herbicide resistance trait has occurred in the case triazine resistance. Herbicide resistance spread of a cytoplasmically inherited trait is

limited to seed dispersal. However, migrant seeds are immediately resistant and spread is determined by the rate of seed survival (Darmency 1996).

While mutation and selection result in the initial appearance of herbicide resistance in a particular geographic area, gene flow is the mechanism for its spread among populations (Jasienuik et al. 1996). In inbreeding species, gene flow is due to seed dispersal. In partially and completely outcrossing species, gene flow is due to pollen movement and outcrossing results in new genotypes.

Crop type and architecture can interfere with pollen movement in outcrossing species (Darmency 1996). Pollen flow and hybridization between common lambsquarters in maize was one tenth that of a pure weed stand (Darmency and Gasquez 1990). Multiple mutation events and founding of resistant populations can look like long distance spread (Darmency 1996). If gene flow rates are greater than the mutation rate then a higher frequency of plants will be resistant to an herbicide prior to the first herbicide application if resistant plants are near (Jasienuik et al. 1996).

Although pollen dispersal has been considered as a major mechanism of inter-population gene flow, seed dispersal may be more important in the spread of a resistant population, especially in self fertilizing species. While pollen dispersal is limited in its movement, blown by the wind, and viability as it dries, seeds may last many years and travel much greater distances. Tumbling plants like kochia can be blown for several miles distributing their seed along the way. Animals and humans move seed as well and seed movement by agricultural equipment can be significant (Maxwell and Ghersa 1992, Stallings et al. 1995, Thill and Mallory-Smith 1997) spreading herbicide resistance. The immigration of susceptible genes into a population can slow development of a resistant

weed population depending on the selection intensity, the mode of inheritance and the rate of immigration of the susceptible genes. Maintaining susceptible genes to manage herbicide resistance is unlikely to be effective; however, because resistance is often dominant, selection is usually high, and immigration rates of as high as 10% do not significantly delay the time it takes for a population to reach a specific frequency of herbicide resistance. Herbicide resistance must be conferred by a recessive allele in a highly outcrossing species for the strategy to work which is unlikely to occur. (Jasienuik et al.1996).

Fitness measures describe the potential evolutionary success of a genotype (Warwick 1991). Resistance genes sometimes have a fitness cost as was found for triazine resistant plants where the photosynthetic ability of the resistant plants was less than susceptible plants (Darmency 1996). In some cases there are subtle effects but not a consistent reduction in plant fitness (Holt and Thill 1994). An accurate quantitative estimate of the relative fitness of resistant and susceptible plants is hard to obtain. Near isogenic resistant and susceptible lines are necessary, experimental conditions should reflect typical field conditions, and traits measured must include the differential survival and reproduction (Jasienuik et al. 1996).

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Chapter 2

The Control of Four Kochia Accessions by Fluroxypyr and Dicamba

David Belles*[1], Philip Westra[1], Scott Nissen[1] and Vanelle Carrithers[2]

[1]Department of Bioagricultural Sciences and Pest Management, Colorado StateUniversity, Fort Collins, CO 80523-1177

[2]Dow AgroSciences, Mulino, OR 97042

The effect of dicamba and fluroxypyr on the control of four kochia accessions was studied. Two accessions were previously determined to be tolerant to dicamba and two accessions were susceptible. The effect of fluroxypyr was unknown. Each accession was treated with five dicamba and fluroxypyr rates. Experiments were conducted in 1999 and 2000 without crop or other weed competition. Visual evaluations were made 3 WAT. Both dicamba resistant accessions were susceptible to fluroxypyr. Dicamba susceptible accessions were also susceptible to fluroxypyr. Regression lines fitted to one resistant and one susceptible line were different as shown by linear contrasts of the slope and lines; however, there was no difference between the lines fitted to data of kochia sprayed with fluroxypyr.

Kochia (*Kochia scoparia* L. Schrad), is a member of the Chenopodiaceae family and part of the native flora of Afghanistan (Holm et al. 1979). First introduced into the United States as an ornamental it became a common weed throughout the western United States infesting cultivated fields, waste lands, roadsides, and gardens (Eberlein and Fore 1984, Whitson et al. 1991). Kochia flowers 8 to 12 weeks after emergence, responding to short days to initiate flowering (Bell et al. 1972). The flowers are inconspicuous and sessile in the upper leaf axils on the plant. The flowers together form short, dense, spikes with bracts (Stubbendick et al. 1997). Although each flower produces only one seed, kochia is a prolific seed producer and one plant can produce over 14,000 seeds (Thill et al. 1993).

Kochia has been effectively controlled with herbicides for many years. Photosystem II, acetolactate synthase inhibitors (ALS), and auxinic herbicides are active on kochia (Christoffoleti 1993). Kochia biotypes resistant to trazine and sulfonylurea herbicides have been identified (Thill 1998). Both trazine and synthetic auxins have been used for over 30 years for weed control; however, 65 species are resistant to triazines but only 24 to synthetic auxins (International Survey of Herbicide Resistant Weeds, 2004).

Resistance to auxinic herbicides was first documented in 1957 where one population of spreading dayflower (*Commelina diffusa* Burm.) in a Hawaiian sugarcane field was reported to be five times more resistant than the susceptible population. Other resistant species have been found in Canada, Spain, France, New Zealand, Indonesia, Malaysia, and the Philippians (Heap 1997). A biotype of kochia resistant to the auxinic herbicide, dicamba, was reported in Montana in 1995 (International Survey of Herbicide Resistant Weeds, 2004).

In 1993, Colorado State University received samples of kochia from Morill, NE that did not respond as expected to dicamba treatments. Some samples survived a dicamba treatment of 1120 g ha^{-1}, four times the recommended dicamba rate for kochia control. Following this observation, over 200 samples were collected from across Colorado. Most of the samples did not exhibit resistance; however, eight samples were less susceptible to dicamba treatments at 280 g ha^{-1}. From 1994 to 1998, kochia samples that exhibited an unexplained lack of control from Colorado, Kansas, Montana, and Nebraska were screened for susceptibility to dicamba. One third of the 50 samples were less susceptible to dicamba. Of the 250 total samples tested 25 exhibited some dicamba tolerance (Howatt 1999). Fluroxypyr is another auxinic herbicide labeled for kochia control. Auxinic herbicides can be divided into four classes based on the type of aromatic groups and the location of the carboxylic acid moiety. These are benzoic acids, pyridines, quinoline carboxylic acids, and phenoxyalkanoic acids (Zheng and Hall 2001). Dicamba is a benzoic acid while fluroxypyr is a pyridine.

Cross resistance is often a problem when a weed develops resistance to an herbicide, other herbicides with the same mode of action also exhibit reduced control. It was hypothesized that the dicamba resistant accessions' response to the auxinic herbicide fluroxypyr, although in another chemical class, would be reduced. An accession of kochia with some resistance to both fluroxypyr and dicamba has been observed in the greenhouse (Dyer et al. 2001). The objective of the study was to determine if kochia accessions resistant to dicamba would also show resistance to fluroxypyr in the field.

Materials and Methods

Kochia Accessions.

A two year study was established at the Colorado State University Agricultural Research Demonstration and Extension Center (ARDEC) in Larimer County, Colorado to compare the efficacy of fluroxypyr and dicamba on four kochia accessions, two resistant and two susceptible. Susceptible accessions were 7710 and Sato. Resistant accessions were Henry and Forsyth. The level of kochia resistance was determined by greenhouse evaluations. Kochia was planted in the field in 1999 and 2000.

Experimental Design.

The experimental was designed as a randomized complete block with each treatment replicated three times. Five rates of dicamba and five rates of fluroxypyr were applied. Dicamba and fluroxypyr at 20, 39, 79, 157, and 314g ha^{-1} were applied in 1999 and 2000. An untreated control was included. Plots were 3 by 4.9 m in 1999 and 1.5 by 5.5 m in 2000. A methylated seed oil was added to each treatment at 1% v/v.

Visual Evaluations.

Herbicide treatments were visually evaluated approximately 21 days after each herbicide application. Kochia control of each accession sprayed with fluroxypyr or dicamba in 1999 and 2000 was modeled using the parabolic function model of the form.

$$y = \beta_0 + \beta_1 x + \beta_2 x^2 + \delta \qquad [1]$$

where, y is the percent kochia injury, x is the herbicide rate in g ha^{-1}, β_0, β_1, and β_2 are the regression coefficients, and δ is the error term under standard linear regression assumptions. Years were not combined because of a significant year and year by treatment interaction. Residual plots of the modeled data showed no significant departure

from assumptions inherent in the regression analysis. Predicted plots were observed for fit and significant parameters are shown (Table 2.1).

Results and Discussion

Visual Evaluation.

Using the regression model, the percent injury was plotted for each kochia accession. The fit of the data to the estimated function varied with accession and is reflected in the R^2 values (Table 2.1). Control of kochia by dicamba was more variable than by fluroxypyr and R^2 values were lower for dicamba. The slope and regression line of the Henry accession differed from the Sato accession in 1999 and 2000 when treated with dicamba (Table 2.2). GR_{50} values were determined based on the regression models (Table 2.3 and 2.4). In 1999, accessions 7710 and Sato were the most susceptible and had GR_{50} values of 135 and 177 g ha^{-1} dicamba, respectively. The Forsyth and Henry accessions required 314 g ha^{-1} or greater for the same level of control indicating dicamba resistance. In 2000, Sato was the most susceptible accession and had a GR_{50} value of 131 g ha^{-1} dicamba. Resistant accessions Forsyth and Henry had GR_{50} values of 312 and >314 g ha^{-1} dicamba, respectively.

A dicamba rate of 540 g ha^{-1} was required to injure a resistant kochia accession to the same degree as 280 g ha^{-1} injured a susceptible accession (Bell et al. 1972). This is similar to our findings on small kochia in 2000 where a rate of 471 g ha^{-1} provided the same control as 280 g ha^{-1} based on the regression model. In 1999, only 50 g ha^{-1} dicamba would be required to control susceptible kochia to the same degree as 471 g ha^{-1} on resistant kochia. The kochia plants themselves may have not become more resistant over the past 30 years but the incidence of the resistant populations may have increased

because of continued selection with dicamba resulting in the recent observations of resistant populations.

Dicamba controlled the Henry kochia accession less than the Sato accession (Figures 2.1 and 2.3). The difference in GR_{50} between Henry and Sato accessions was at least 137 and 183 g ha^{-1} dicamba and R/S ratios were at least 1.7 and 2.4 in 1999 and 2000, respectively. The GR_{50} values of dicamba inbred R and S kochia lines, developed under pollen isolation conditions for two generations, had ED_{50} values of 143 and 31 g ha^{-1} and an R/S ratio of 4.6 when treated with dicamba (Cranston et al. 2001)

Different levels of resistance can be observed in different studies and related to varying environmental conditions. In two greenhouse studies resistance levels were only 3 fold in the winter versus 11 to 35 fold in the summer (Fuerst et al. 1996). Our results were more consistent; however, if large variations can be observed in the greenhouse where conditions are relatively constant variation in the field may be even greater and environmental conditions more important. The interaction between the environment and resistance level should be studied.

Kochia control with fluroxypyr was similar across all kochia accessions. Regression lines for kochia accessions Sato and Henry treated with fluroxypyr were not different in 1999 or 2000 and showed no evidence of fluroxypyr resistance (Figures 2.2 and 2.4). In contrast to treatment with dicamba, differences between GR_{50} values of Henry and Sato accessions treated with fluroxypyr were 11 and 5 g ha^{-1} (Tables 2.3 and 2.4). R/S ratios were 1.1 and 1.03 in 1999 and 2000, respectively, indicating that the dicamba resistant accession was susceptible to fluroxypyr. In another study, fluroxypyr applied at 140 g ha^{-1} provided 88 to 99% of four inbred dicamba tolerant kochia lines.

Dicamba control ranged from 59 to 73% on the same lines at 140 g ha^{-1} (Nandula and Manthey 2002). The R/S ratio of picloram resistant yellow starthistle when treated with dicamba was 4.4 indicating that yellow starthistle resistant to picloram was cross resistant to dicamba (Fuerst et al. 1996). Although picloram and fluroxypyr are both pyridines the dicamba tolerant kochia in this study did not show cross resistance to fluroxypyr.

Examples of kochia accessions resistant to fluroxypyr and dicamba do exist (Dyer et al. 2001, Goss and Dyer 2003). An accession resistant to both dicamba and fluroxypyr had an intermediate phenotype of root growth inhibition and was only slightly resistant to dicamba. It was suggested that the biotype resistant to both dicamba and fluroxypyr had a different resistance mutation than the dicamba resistant biotype (Goss and Dyer 2003). This conclusion can agree with our result that the dicamba resistant accession was susceptible to fluroxypyr if our resistant accessions possess only the mutation for dicamba tolerance.

Fluroxypyr was effective on the dicamba resistant lines tested and could be used for dicamba resistant kochia control if another mutation for fluroxypyr resistance has not also been selected for in the same kochia population.

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Table 2.1. Parameter estimates, standard errors, probability of greater (t), and adjusted R^2 values for regression models of dicamba and fluroxypyr sprayed on Sato and Henry accessions in 1999 and 2000.

	Sato					
	Dicamba			Fluroxypyr		
	B_0	B_1	B_2	B_0	B_1	B_2
Control 1999						
Estimate	-2.54	0.50	<0.00	6.07	0.51	<0.00
Std err	6.72	0.15	<0.00	8.48	0.17	<0.00
Pr>|t|	0.7110	0.0042	0.0208	0.4850	0.0090	0.1560
R^2		0.54			0.68	
Control 2000						
Estimate	-5.38	0.56	<0.00	-3.65	0.42	<0.00
Std err	7.90	0.16	<0.00	7.57	0.15	<0.00
Pr>|t|	0.506	0.0033	0.0490	0.6367	0.0152	0.2828
R^2		0.66			0.70	

	Henry					
	Dicamba			Fluroxypyr		
	B_0	B_1	B_2	B_0	B_1	B_2
Control 1999						
Estimate	0.72	0.12	<0.00	9.70	0.43	0.00
Std err	4.38	0.10	<0.00	8.36	0.16	<0.00
Pr>|t|	0.8717	0.2416	0.6335	0.2632	0.0167	0.2505
R^2		0.34			0.63	
Control 2000						
Estimate	-1.32	0.16	<0.00	-4.20	0.41	<0.00
Std err	4.25	0.086	<0.00	4.04	0.08	<0.00
Pr>|t|	0.7599	0.0823	0.8425	0.3153	0.002	0.1522
R^2		0.73			0.92	

Table 2.2. Contrasts between slops and lines of accessions Sato and Henry in 1999 and 2000, F values and probability of greater F.

	Contrasts							
	Slopes Sato vs. Henry				Lines Sato vs. Henry			
Year	1999		2000		1999		2000	
Treatment	dic	flu	dic	flu	dic	flu	dic	flu
F value	4.01	0.14	4.01	0.00	3.59	0.09	4.05	0.29
Pr > F	0.0492	0.7112	0.0493	0.9492	0.0178	0.9663	0.0105	0.8293

Table 2.3. GR_{50} and adjusted R^2 values based on regression equations for dicamba and fluroxypyr in 1999.

1999	Dicamba		Fluroxypyr	
Accession	GR_{50}	R^2	GR_{50}	R^2
	g ha^{-1}		g ha^{-1}	
7710	135	.6088	92	.7212
Henry	>314	.3385	112	.6251
Forsyth	>314	.3681	106	.6182
Sato	177	.5384	101	.6811

Table 2.4. GR_{50} and adjusted R^2 values based on regression equations for dicamba and fluroxypyr in 2000.

2000	Dicamba		Fluroxypyr	
Accession	GR_{50}	R^2	GR_{50}	R^2
	g ha^{-1}		g ha^{-1}	
7710	238	.6938	119	.8987
Henry	>314	.6991	155	.9168
Forsyth	312	.5462	131	.9026
Sato	131	.6560	160	.7045

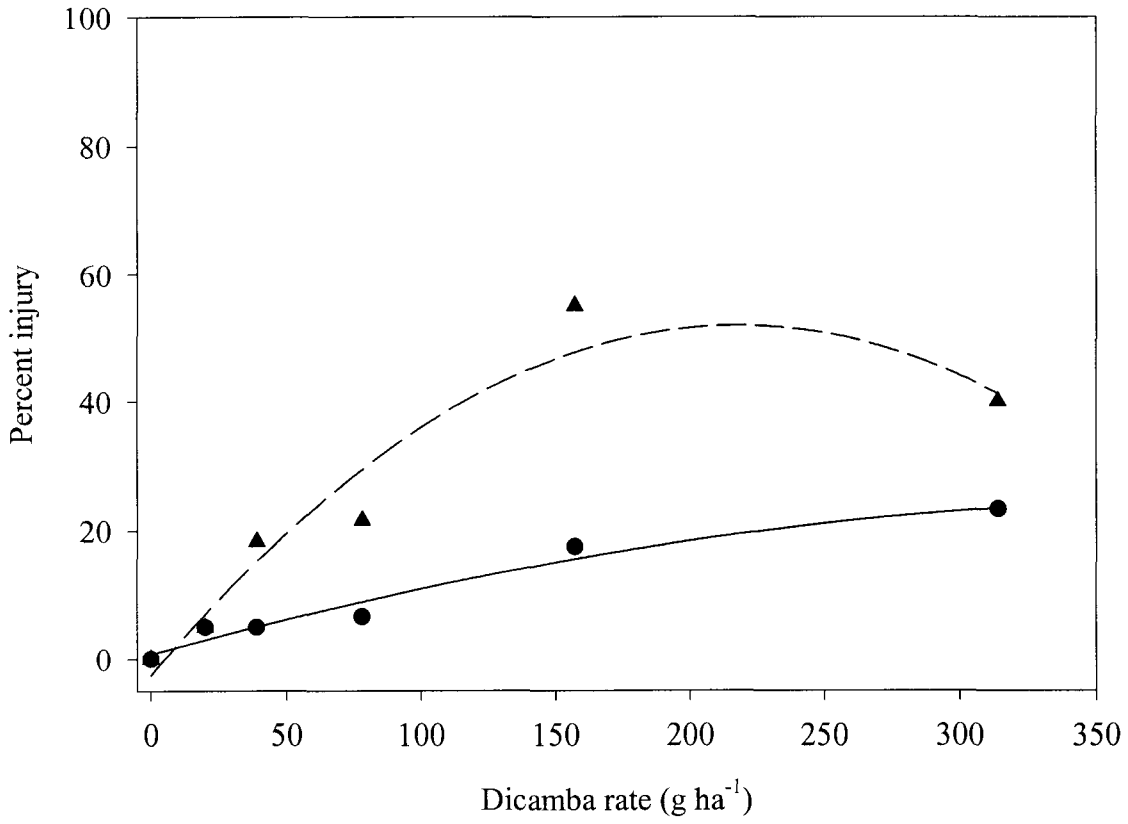

Figure 2.1. The relationship between herbicide injury and dicamba rate on susceptible (dashed line, ▲) and resistant (solid line, ●) kochia (Sato and Henry) in 1999. Estimated equations for lines (kochia accessions) are $\hat{y} = -2.54 + (.50)(\text{rate}) + (<0.0)(\text{rate})^2$ for Sato ($R^2 = .54$) and $\hat{y} = 0.72 + (.12)(\text{rate}) + (<0.0)(\text{rate})^2$ for Henry ($R^2 = .34$).

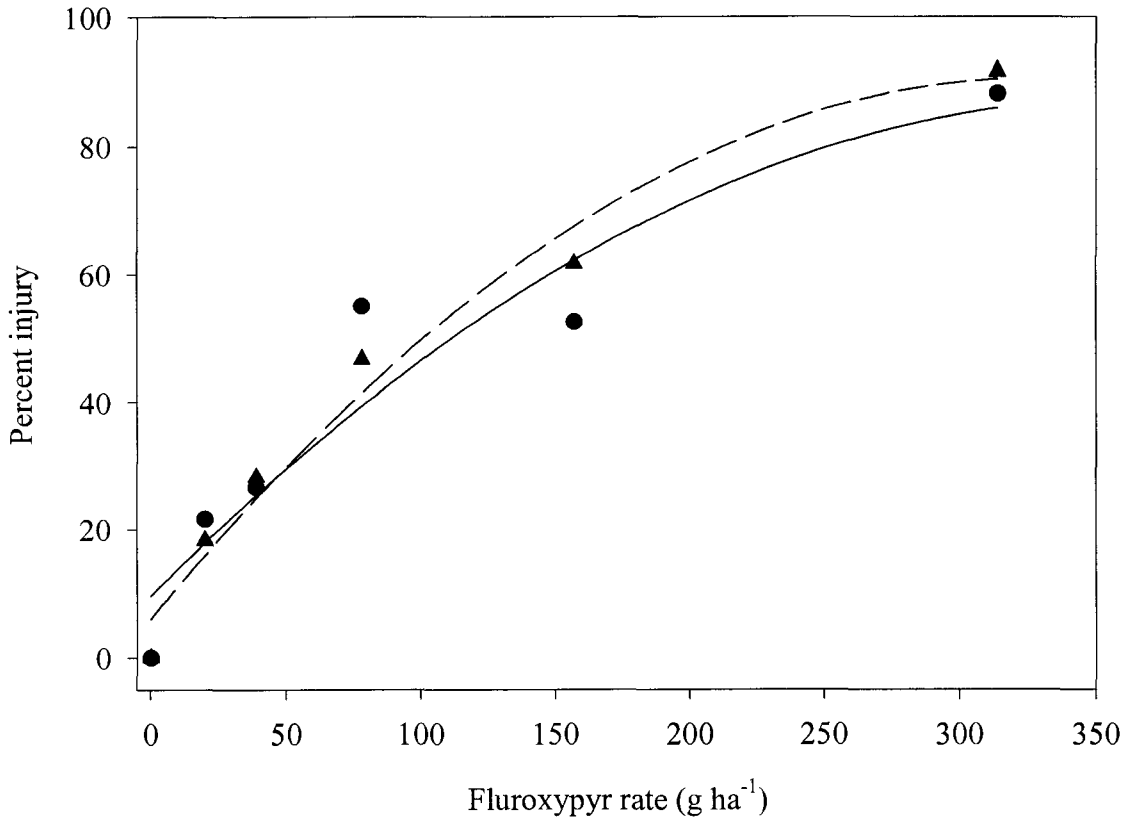

Figure 2.2. The relationship between herbicide injury and fluroxypyr rate on susceptible (dashed line, ▲) and resistant (solid line, ●) kochia (Sato and Henry) in 1999. Estimated equations for lines (kochia accessions) are $\hat{y} = 6.07 + (.51)(rate) + (<0.0)(rate)^2$ for Sato ($R^2 = .68$) and $\hat{y} = 9.69 + (.43)(rate) + (<0.0)(rate)^2$ for Henry ($R^2 = .63$).

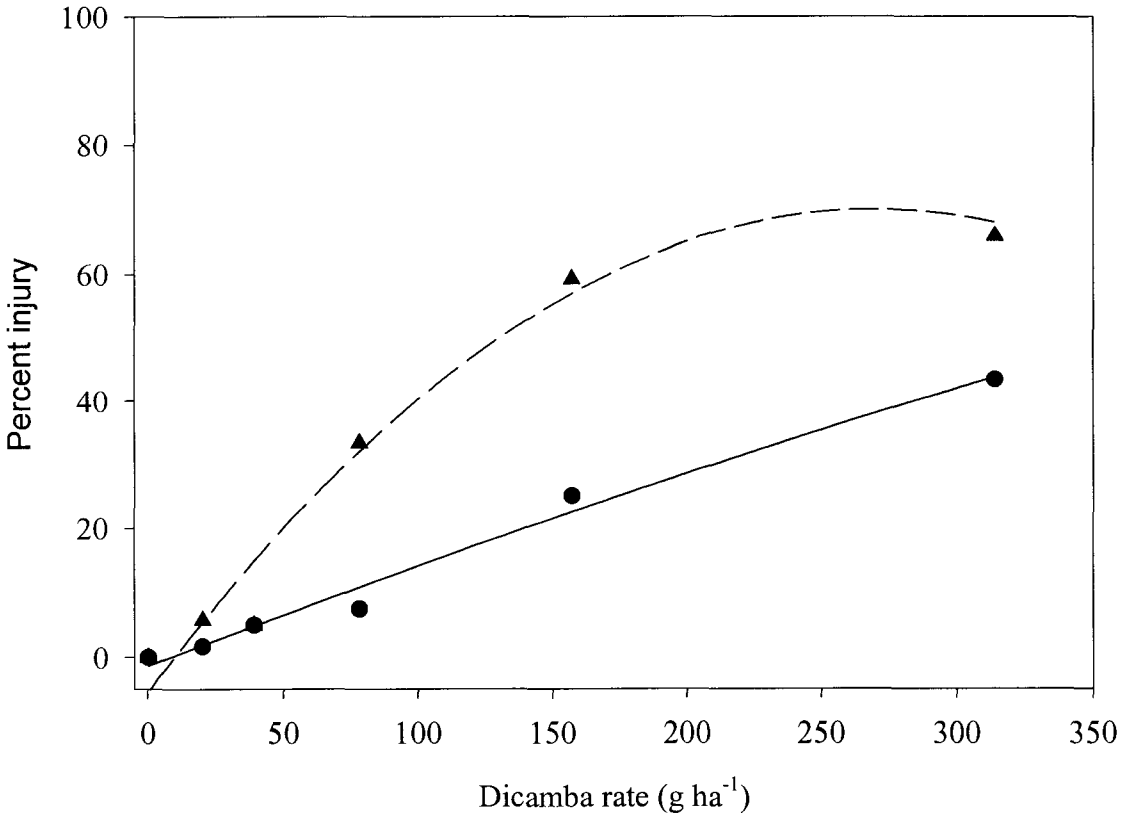

Figure 2.3. The relationship between herbicide injury and dicamba rate dicamba on susceptible (dashed line, ▲) and resistant (solid line, ●) kochia (Sato and Henry) in 2000. Estimated equations for lines (kochia accessions) are $\hat{y} = -5.38 + (.56)(\text{rate}) + (<0.0)(\text{rate})^2$ for Sato ($R^2 = .66$) and $\hat{y} = -1.32 + (.16)(\text{rate}) + (<0.0)(\text{rate})^2$ for Henry ($R^2 = .73$).

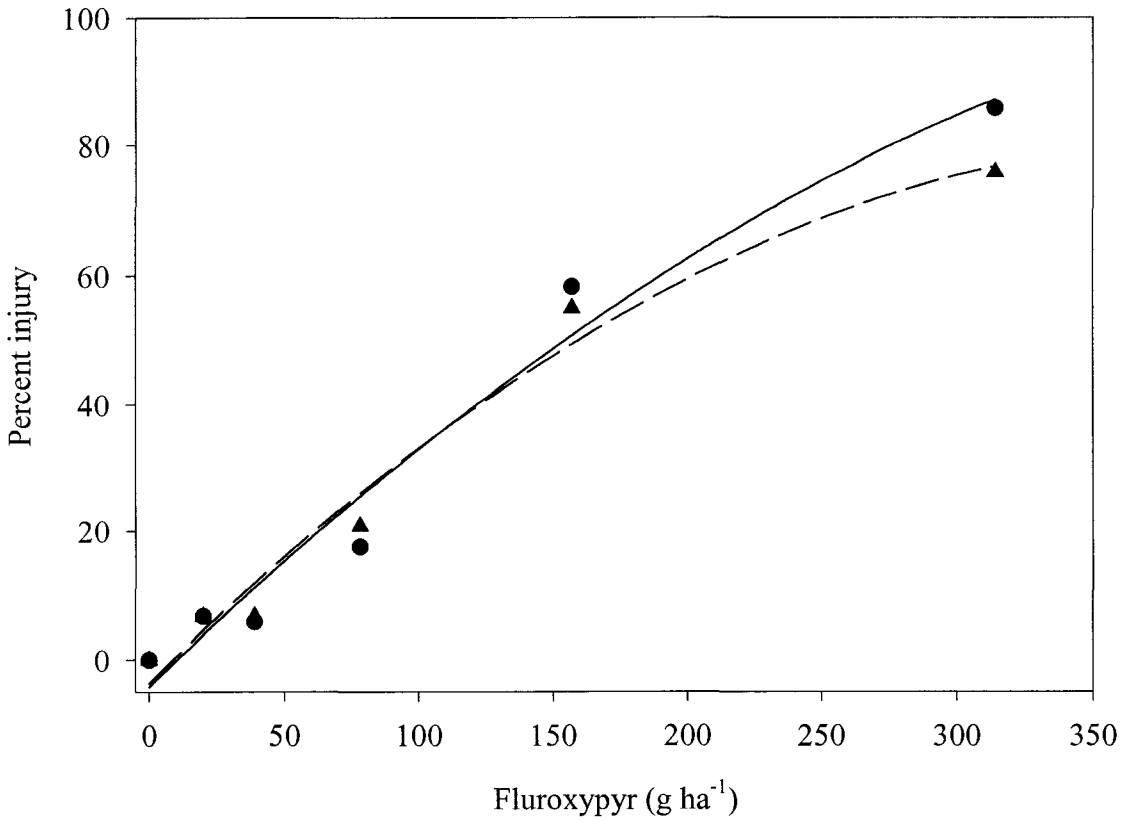

Figure 2.4. The relationship between herbicide injury and dicamba rate dicamba on susceptible (dashed line, ▲) and resistant (solid line, ●) kochia (Sato and Henry) in 2000. Estimated equations for lines (kochia accessions) are $\hat{y} = -3.65 + (.42)(\text{rate}) + (<0.0)(\text{rate})^2$ for Sato ($R^2 = .70$) and $\hat{y} = -4.20 + (.41)(\text{rate}) + (<0.0)(\text{rate})^2$ for Henry ($R^2 = .92$).

Chapter 3

Ethylene production by dicamba resistant and susceptible kochia (*Kochia scoparia*) treated with dicamba and fluroxypyr

David S. Belles*[1], Scott J. Nissen[1], Philip Westra[1], Christopher Lawrence[2], Kirk Howatt[3]

[1]*Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins, CO 80523-1177*

[2]*Virginia Bioinformatics Institute Blacksburg, VA 24061*

[3]*Department of Plant Sciences, North Dakota State University Fargo, ND 58105*

*Corresponding author

Ethylene is produced in many plants in response auxinic herbicides applications. Wild mustard and yellow starthistle resistant to auxinic herbicides produce less ethylene than susceptible plants. Experiments were conducted to determine if dicamba resistant kochia produced less ethylene than susceptible kochia, to determine if any decrease in ethylene production was related to dicamba herbicide symptoms, and to determine if there was a relationship between ethylene production and fluroxypyr susceptibility. Dicamba resistant kochia treated with 280 g ha^{-1} dicamba produced 459 nl g fw^{-1} less ethylene than susceptible kochia 24 HAT. Ethylene production increased faster in susceptible than resistant kochia as dicamba rate increased. Dicamba resistant and susceptible kochia treated with fluroxypyr produced similar amounts of ethylene as herbicide rate increased. GR_{50} values for susceptible and resistant kochia treated with dicamba were 45 and 1331 g ha^{-1}, respectively. GR_{50} values for susceptible and resistant kochia treated with fluroxypyr were 10 and 34 g ha^{-1}, respectively. The ethylene inhibitor AOA inhibited ethylene production but did not reduce dicamba or fluroxypyr symptoms. Senescence was delayed in ethylene insensitive tobacco when treated with dicamba but not fluroxypyr.

Ethylene, C_2H_4, is an important plant hormone produced in small amounts in almost all higher plants. In 1931, ethylene was discovered to cause epinasty in plants and stimulate root growth (Zimmerman et al. 1931 and Zimmerman and Hitchcock 1933). If only a single leaf was exposed the entire plant was affected. Ethylene is now recognized to regulate fruit ripening, leaf defoliation, activate defense chemicals, and perhaps enable plants to communicate with each other (Wink 1997, Deikman, 1997).

Higher indole-3-acetic acid (IAA) concentrations increase ethylene levels by increasing the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) from S-adenosyl-L-methionine (SAM) (Yang and Hoffman 1984, Yoshii and Imaseki 1981, John 1997). IAA, a plant hormone, causes many plant responses that include: cell elongation, cell division, cell differentiation, root initiation, tropic responses, leaf senescence, cell and organ polarity, and wound responsiveness (Hall 1997). Some of these responses may be due to interactions with ethylene and other plant hormones. The direct effects of ethylene and auxin are further complicated by the fact that auxin induces ethylene production.

Dicamba and fluroxypyr are synthetic auxins with herbicidal activity at high concentrations. The activities of dicamba and fluroxypyr are believed to mimic auxin, but cause a much greater and severe response leading to plant death in susceptible species. Dicamba and fluroxypyr promote similar symptoms in kochia; however, they are distinct in their chemical structure. Dicamba is a benzoic acid, while fluroxypyr is a pyridine. The precise herbicidal target for auxinic herbicides is unknown. The induction of ethylene production by auxinic herbicides may contribute to herbicidal effects. Severe

epinasty was observed in susceptible wild mustard but not resistant mustard following picloram applications. Picloram is an auxinic herbicide structurally related to fluroxypyr. In susceptible biotypes, ACC synthase levels, ACC, 1-malonyl amiocyclopropane-1-carboxylic acid (MACC), and ethylene increased after picloram application and both susceptible and resistant biotypes exhibited epinasty when fumigated with 120 $\mu l\ l^{-1}$ ethylene. Differences in the phenotypic response to picloram between resistant and susceptible biotypes may be due to differences in de novo synthesis of ACC synthase in the ethylene biosynthesis pathway; however, ACC synthase activity was not impaired in resistant plants either (Hall et al 1993). Different levels of ethylene synthesis are not due to differential sensitivity of the ACC synthase enzyme in wild mustard (Hall et al. 1993).

The effect of picloram on resistant and susceptible yellow starthistle and the role of ethylene was evaluated (Sabba et al. 1998). Ethylene production in susceptible plants was 250% greater than in resistant plants; however, ethylene production did not account for all of the epinasty observed in susceptible plants. When ethylene production was inhibited in picloram treated plants, epinasty was only reduced by 20% and ethephon, an ethylene releasing compound, caused only a small amount of epinasty when applied. In yellow starthistle, ethylene production induced by picloram played only a small part in herbicidal action and resistance to picloram was not due to the inability of the resistant biotype to produce ethylene (Sabba et al. 1998). An altered site for auxinic herbicide binding such as an auxin binding receptor could be a possible resistance mechanism.

The effect of ethylene on dicamba susceptible and tolerant kochia was first reported by Howatt (1999). Dicamba treated susceptible kochia produced an average of 2.5 times more ethylene than resistant kochia; however, ethylene produced by ethephon

at levels similar to those observed after dicamba treatments did not produce epinastic symptoms. This suggested that in kochia treated with dicamba, ethylene production is not responsible for the herbicidal effect or plays only a minor role (Howatt 1999). Ethylene also played only a small part in the herbicidal effect of picloram on yellow starthistle (Sabba et al. 1998).

Ethylene inhibitors have often been used to study ethylene by eliminating ethylene effects. The use of ethylene inhibitors could be used to further evaluate the effect of ethylene on auxinic herbicide activity in kochia. If ethylene production of a susceptible kochia accession treated with dicamba could be eliminated with an ethylene inhibitor and the corresponding herbicide injury symptoms eliminated it would support an important role for ethylene in dicamba's herbicidal action. Some symptoms may be due to ethylene, while others are due to other herbicide effects. Ethylene may also have other effects besides epinasty which may be more important in the resistance of kochia to dicamba. One of these may be senescence, programmed cell death, apoptosis. Ethylene is known to be involved in promoting fruit senescence as well as tissue senescence through programmed cell death or apoptosis (Yamada et al. 2001, John et al. 1995, Grbić and Bleeker 1995).

Cross resistance between different auxinic herbicides is a concern. Ethylene production should indicate whether a kochia biotype resistant to dicamba will also be resistant to another auxinic herbicide. An increase in ethylene production in a dicamba resistant kochia should correspond to susceptibility to a different auxinic herbicide.

We hypothesized that resistant kochia would produce less ethylene than susceptible kochia when treated with dicamba and that the level of ethylene production

would correspond to a decrease in kochia biomass. However, when treated with fluroxypyr ethylene would be produced and the plant would be susceptible to fluroxypyr treatments. We postulated that ethylene inhibitors would eliminate the symptoms of dicamba and ethylene insensitive tobacco would be less affected by dicamba treatments than normal tobacco. Dose response, time course, and ethylene inhibitor studies were conducted to evaluate ethylene production by dicamba resistant and susceptible kochia. An ethylene inhibitor was used to separate the effects of ethylene and auxin on herbicide resistance. Ethylene production after treatment with fluroxypyr was measured to determine if cross resistance could be measured by ethylene production. The response of insensitive tobacco mutants to dicamba and fluroxypyr was also evaluated.

Materials and Methods

Plant Material.

Dicamba susceptible and resistant kochia accessions were identified by treating each accession with dicamba and comparing the injury to a known susceptible population. Resistant seed was collected and self pollinated for seven generations to produce a uniform resistant population. Using this resistant seed a study was conducted to measure the amount of ethylene produced by dicamba susceptible and resistant kochia over time. Kochia was planted in individual 6 cell pack flat insert containers[1]. Each individual cell was 3 cm by 3 cm by 3 cm. The cells were filled with dry Metro Mix 200[2]. Seeds were sprinkled on the surface of the soil and covered with 0.3 cm of dry potting mix. Flats were then watered. When all the plants emerged they were thinned to

one plant per cell. Plants were grown in the greenhouse under a 14/10 h day/night photoperiod supplemented with 400 w sodium halide lights. Day night temperatures were 24 and 18 C, respectively. These greenhouse conditions and planting procedures were used for all experiments.

Ethylene Measurement.

Ethylene production was measured by GC-FID using a HP5890 gas chromatograph[3] fitted with a Carboxen 1006-PLOT column[4] with an internal diameter of 0.53 mm. The oven temperature was an isothermic 100 C for the entire run. The detector and injector temperatures were 250 C. He, H and air flows were set at 10, 20, and 390 ml min^{-1}, respectively. The average run time was 4.2 min. A 10 ppm ethylene in air standard[5] was used as a standard to quantify ethylene produced. A standard was injected before and after each experiment and after each replication was completed. A 200 µl head space sample was injected. The area under each peak was integrated using the HP-Chromatography software.

Time Course Experiments.

When kochia plants were 5 to 7 cm tall dicamba was applied at 280 g ha^{-1} using a spray chamber calibrated to deliver 141 L ha^{-1} at 97 kPa. Five resistant and susceptible plants were harvested 0, 6, 12, 24, 48, and 72 HAT. Plants were harvested by cutting the stem at the soil surface. Immediately after harvesting, the plants were weighed, placed in 30 ml glass test tubes and sealed with a rubber septum. The fresh weight was used to

standardize ethylene production on a gram fresh weight basis. Plants were incubated in the test tubes for two hours at room temperature under fluorescent room lights.

Dose Response Experiments.

Plant Dry Weight.

A second dose response study was conducted to correlate the dry biomass of the kochia with the ethylene produced. Resistant and susceptible seeds were planted and grown by the same procedure and conditions previously mentioned. Plants were sprayed when they were approximately 11 cm tall following the same procedure as the ethylene production experiments. Dicamba was applied at 11, 56, 280, 1400, and 7000 g ha^{-1} and fluroxypyr was applied at 1, 6, 28, 140 and 700 g ha^{-1}. An untreated control was included. Three weeks after treatment the plants were harvested, dried at 60 C for seven days and weighed. Five plants were harvested in each treatment and the experiment was repeated.

Ethylene Production.

A dose response study was conducted to evaluate the effect of increasing dose on ethylene production in resistant and susceptible kochia. Plants were treated with 11, 56, 280, 1400, and 7000 g ha^{-1} dicamba or 1, 6, 28, 140, and 700 g ha^{-1} fluroxypyr. An untreated control was included. All plants were harvested and ethylene determined 48 HAT.

Ethylene Production Inhibition.

To evaluate the importance of ethylene to auxin herbicide activity, plants were pretreated with the ethylene inhibitor, aminooxy acetic acid (AOA), an inhibitor of 1-amino-1-cyclopropane carboxylic acid (ACC) synthase. Two days before herbicide treatment individual plants were removed from the soil and their roots carefully washed. They were placed into 15 ml scintillation vials containing 10 ml MS media at 4.3 g L^{-1}, with 100, μM AOA. Non-absorbent cotton was wrapped around each stem to hold the plant upright and to prevent herbicide from entering the growth media when the plants were sprayed. Samples were placed in a growth chamber with 22/20 C day night temperatures and an 18 h photoperiod. After 2 days the AOA was replaced with fresh MS media and fresh MS media was added to the vials of the other samples. Plants were then sprayed with either 280 g ha^{-1} dicamba or 140 g ha^{-1} fluroxypyr and placed back in the growth chamber for two more days. Plants were then harvested by cutting off the stem where the root tissue began, weighed, placed in 30 ml glass vials, capped, and ethylene production was measured.

A dose response study with AOA concentrations of 12.5, 25, 50 and 100 μM AOA was conducted on dicamba susceptible plants and the ethylene production measured after two days. One set of plants was sprayed with dicamba at 280 g ha^{-1} and another was left untreated. There were five plants per treatment. Another set of susceptible plants were allowed to grow for 7 days after dicamba treatment at 280 g ha^{-1} in 0, 12.5, 25, and 50 μM AOA. The dicamba induced epinasty and ethylene production was observed and compared to dicamba untreated plants.

Ethylene Mutants in Tobacco.

A final study evaluated the effect of dicamba and fluroxypyr on ethylene insensitive tobacco. TetR tobacco containing the etr1 gene mutation and Samsun NN tobacco, the wild type tobacco, were obtained and planted in rows in 24 by 24 cm flats using potting soil[2]. After emergence plants were thinned to 6 plants per row. When the tobacco had approximately 5 leaves they were treated with 280 g ha^{-1} dicamba or 140 g^{-1} fluroxypyr using a spray chamber calibrated to deliver 141 L ha^{-1} 207 kPa. Visual evaluations were made 14 days after treatment.

Statistical Analysis.

Statistical computations were carried out using SAS/STAT (2001). Analysis of variance (ANOVA) was used to determine inhibitor effect, plant biomass, control, and interaction significance for all measured response variables. Tukey's studentized range test and standard errors (SE) were used to compare inhibitor treatment effects. Analysis of variance was used to determine time, herbicide rate, kochia accession and time or herbicide rate and kochia accession interaction significance for ethylene production. Regression analysis was used to determine the effect of time, and kochia accession on ethylene production. Residual mean square and predicted sum of squares were examined and data fit to a linear model.

Regression analysis was used to determine the effect of herbicide dose on ethylene production and kochia dry weight. Dose response residual mean square and predicted sum of squares were examined and a log-logistic non-linear curve was fitted to data for each herbicide (Seedfeldt et al. 1995).

Results and Discussion

Time Course Experiments.

Cutting the plants at the soil surface did not cause an increase in ethylene sufficient to mask any herbicide effect as evidenced by the small amount of ethylene produced in the untreated plants. No ethylene was produced 0 HAT. Ethylene production by resistant kochia was slower than susceptible kochia and less ethylene was produced by the resistant kochia 6, 12, 24, 48, and 72 HAT. Ethylene production as a function of time was modeled using a linear model. The linear model fit the susceptible kochia data ($R^2 = .82$). Model residual plots showed no significant pattern and both model parameters were significant. The linear model did not fit the resistant kochia data as well ($R^2 = .71$) (Figure 3.1). The slopes of the two regression lines were different indicating different ethylene production rates. If the experiment had been terminated 24 HAT the slope and intercept of the linear regression line for the resistant kochia would not be significant indicating that no ethylene was produced by resistant kochia during this time. At 6, 12, and 24 HAT the amount of ethylene produced was less than 150 nl g fw^{-1} and some resistant plants still produced no ethylene even 24 HAT; however, ethylene production in susceptible kochia increased linearly during this time. Resistant kochia began to produce ethylene 24 HAT and the ethylene production rate 24 to 72 HAT by resistant kochia was the same as susceptible kochia.

Little to no ethylene was produced by resistant kochia at 6, 12 and 24 HAT, but by 12 HAT 203 nl g fw^{-1} was produced by susceptible kochia compared to 56 nl g fw^{-1} by the resistant kochia. The greatest difference in ethylene production between resistant and susceptible kochia was at 24 HAT. The resistant kochia produced ethylene at about the same rate as the susceptible kochia after 24 h and the difference in the ethylene level

between resistant and susceptible accessions did not increase. Ethylene production was still increasing in both resistant and susceptible kochia 72 HAT.

The continued increase in ethylene production found in the current study differs from reports that ethylene production peaks a few hours after herbicide treatment. When picloram was applied to resistant and susceptible yellow starthistle the increase in ethylene production peaked in susceptible plants 24 HAT. Resistant plants did not produce ethylene even 48 HAT (Valenzuela-Valenzuela et al. 2001). Fuerst et al. (1996) showed an initial increase in ethylene production in yellow starthistle that decreased after 6 h. A peak in ethylene production by kochia treated with 140 g ha^{-1} dicamba was observed 48 HAT (Howatt 1999). Dicamba applied at a higher rate of 280 g ha^{-1} may have delayed the decrease in ethylene production in our study beyond 72 h. Ethylene production began to decline only after 96 h in susceptible wild mustard treated with picloram (Hall et. al. 1993), while the resistant wild mustard did not produce ethylene 96 HAT. Resistant kochia evaluated in the current study began producing ethylene 24 HAT. The peak in ethylene production may indicate a physiological change within the plant signaling the beginning of senescence. The height of the peak may also be important. If a critical ethylene production threshold is not reached epinasty or plant senescence may not be triggered. Two senescence pathways have been identified; one involving caspases requires low levels of ethylene and another caspase independent pathway that is activated at high ethylene levels (de Jong et al. 2002). After 72 h, 575 nl g^{-1} fw was produced by the resistant kochia, while 1013 nl g^{-1} fw was produced by the susceptible kochia.

Dose Response Experiments.

Plant Dry Weight.

Kochia dry weight as a percent of the control was plotted against ethylene production over increasing dicamba dose and fit to a log logistic model to calculate GR_{50} values (Figure 3.2B). GR_{50} values were 1331 g ha^{-1} and 45 g ha^{-1} for resistant and susceptible kochia, respectively, producing an R/S ratio of 30. Dry weight did not decrease until ethylene production increased for both resistant and susceptible kochia and greater ethylene production was related to a greater reduction in dry weight in the susceptible compared to the resistant biotype.

Dicamba resistant and susceptible kochia were susceptible to fluroxypyr (Figure 3.3). Epinastic symptoms were greater than when treated with dicamba and plants senesced more rapidly. GR_{50} values for susceptible and resistant kochia treated with fluroxypyr were 10 and 34 g ha^{-1}, respectively an R/S ratio of 3.4.

Ethylene Production.

Ethylene production increased as dicamba dose increased from 0 to 7000 g ha^{-1} and ethylene production in susceptible kochia increased faster than in resistant kochia (Figure 3.2A). Data from repeated experiments were not combined because of significant interactions and representative data are shown (Figure 3.2).

Ethylene production as a function of dicamba rate was modeled using a non-linear log-logistic model (Figure 3.2). More ethylene was produced by susceptible kochia than resistant kochia as dicamba rate increased and there was a difference at all rates except 11 g ha^{-1}. Dicamba induced ethylene production in resistant kochia but it was always lower than susceptible kochia. Ethylene production by susceptible kochia started to level off at 7000 g ha^{-1} but ethylene production by resistant kochia continued to increase. In contrast, ethylene production was not induced when clopyralid was applied to resistant wild

mustard (Hall et al. 1993). Dicamba resistance based on reduced ethylene production is not a complete resistance. If enough dicamba was applied ethylene was produced and this production proceeded in a dose dependent manner.

Ethylene production by dicamba resistant kochia was not consistently lower than susceptible kochia at the fluroxypyr rates tested (Figure 3.3A). Although less ethylene was produced by dicamba resistant kochia at 6 g ha^{-1}, more ethylene was produced than dicamba susceptible kochia at 700 g ha^{-1} fluroxypyr. Ethylene production did not show cross resistance between dicamba and fluroxypyr and more ethylene was produced by both resistant and susceptible lines when sprayed with fluroxypyr compared to dicamba.

Cross resistance between auxinic herbicides does exist. Yellow starthistle was found to be resistant to dicamba, fluroxypyr, clopyralid, and picloram (Fuerst et al. 1996). Ethylene production increased in susceptible but not resistant yellow starthistle when sprayed with either clopyralid or picloram. Ethylene production was not reported for yellow starthistle treated with triclopyr or 2,4-D which the yellow starthistle was susceptible to (Valenzuela-Valenzuela et al. 2001).

Fluroxypyr resistant and fluroxypyr/dicamba cross resistant kochia accessions have been identified although the fluroxypyr/dicamba resistant accession was only slightly resistant to dicamba (Dyer et al.2001, Goss and Dyer 2003). It would be interesting to measure ethylene production in these accessions and compare it to herbicide resistance levels.

Ethylene Production Inhibition.

Susceptible kochia treated with 280 g ha^{-1} dicamba produced more ethylene than the resistant kochia accession (Figure 3.4). AOA applied alone at 100 μM did not increase ethylene production while pretreatment of AOA two days before dicamba application reduced the amount of ethylene produced. Ethylene production was also reduced by 25 and 50 μM AOA.

Pretreatment with 12.5 μM AOA did not reduce ethylene production in susceptible plants sprayed with dicamba. Only 50 and 100 μM AOA reduced ethylene production to levels observed in untreated plants. Pretreatment with AOA and a corresponding reduction in ethylene production did not reverse the epinastic symptoms caused by dicamba; however, symptom development was delayed. Therefore, elimination of ethylene production by AOA suggests ethylene is not involved in the epinastic symptoms caused by dicamba. Sabba et al. (1998) and Hall et al. (1993) concluded that ethylene was involved in the herbicidal action of picloram on wild mustard but not on yellow starthistle. When exposed to exogenous ethylene only minor symptoms developed in yellow starthistle; however, significant epinasty resulted when wild mustard was exposed to exogenous ethylene.

Ethylene Mutants in Tobacco.

Ethylene inhibitors have other effects on the plant besides inhibiting ethylene production, confounding results of inhibitor experiments. An ethylene insensitive transgenic plant will not have this problem. Therefore, ethylene insensitive tobacco, ETR1, was treated with dicamba and fluroxypyr. The effect on ethylene insensitive tobacco was compared to the effect on a normal tobacco line Samsun NN. Although

there was no difference in tobacco biomass as measured by dry weight one month after treatment. Fourteen DAT, dicamba controlled ethylene insensitive and normal tobacco lines 82 and 94%, respectively. Fluroxypyr controlled the ethylene insensitive and normal lines 93 and 98%, respectively (data not shown). Ethylene insensitive plants treated with dicamba stayed green longer than the dicamba treated normal line or either line treated with fluroxypyr. Ethylene insensitive plants treated with fluroxypyr all became chlorotic and necrotic before those treated with dicamba. Also, in the first one to two days after herbicide application the ethylene insensitive tobacco showed less epinastic symptoms when treated with dicamba compared to the other herbicides. This was generally characterized by epinasty only at the base of the leaf petioles, causing the leaves to bend upward, without the typical epinasty of the leaf or curvature of the petiole observed in the Samsun NN tobacco or when treated with fluroxypyr. Because the TetR tobacco is insensitive to ethylene it was assumed that both herbicides would have reduced symptomology in correlation with the symptoms caused by ethylene. However, the ethylene insensitivity did not seem to inhibit the ability of fluroxypy to injure the TetR tobacco plants. This may mean that fluroxypyr induces senescence by another mechanism, or is perceived by the plant differently than dicamba, in addition to increasing ethylene production.

In this study, ethylene production proved a good indicator of auxinic herbicide resistance in kochia. Resistance and susceptibility to dicamba and fluroxypyr correlated with the level of ethylene produced after herbicide treatment. However, elimination of ethylene production by AOA did not eliminate herbicide symptoms, although, there was a brief delay in their development. Plants could not be continuously grown and fed AOA

long enough to determine if senescence was delayed in susceptible kochia when ethylene production was eliminated; however, senescence was delayed in ethylene insensitive tobacco plants treated with dicamba but not fluroxypyr indicating ethylene may be the senescence trigger activated by dicamba. Fluroxypyr may have other mechanisms to trigger senescence and a slightly different mode of action or may have a slightly different receptor or regulation of plant signaling pathways than dicamba in kochia.
This may be one reason for the lack of cross resistance to fluroxypyr as well as for fluroxypyr's ability to cause ethylene production in the dicamba resistant line.

Sources of Materials

[1]Greenhouse flat inserts, East Jordan Plastics, Inc. P.O. Box 575 East Jordan, MI 49727.

[2] Metro Mix 200, Scotts-Sierra Horticultural Products Co. 14111 Scottslawn Rd., Marysville, OH 43041.

[3]HP5890 Gas chromatograph, Agilent, 395 Page Mill Rd., P.O. Box #10395, Palo Alto, CA 94303.

[4]Carboxen 1006 PLOT column, Supelco, Supelco Park Bellefonte, PA 16823.

[5] Ethylene standard, Scotts Specialty Gases, Supelco, Supelco Park, Bellefonte, PA 16823.

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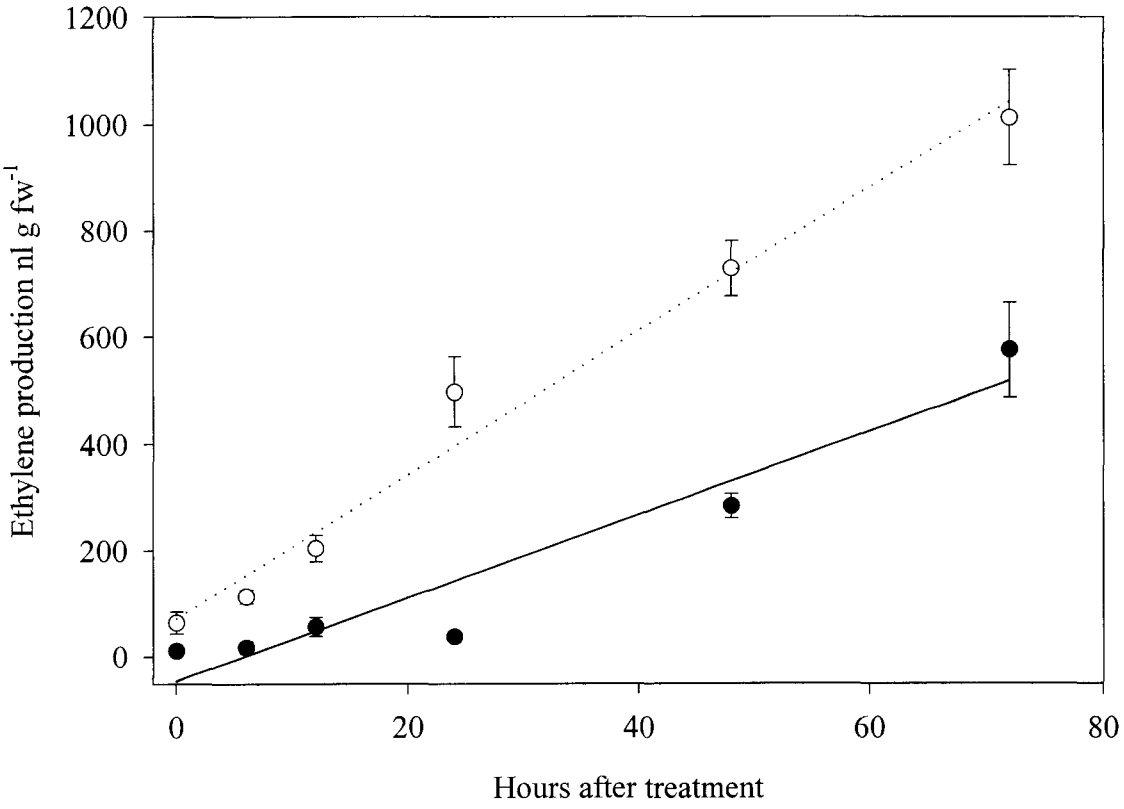

Figure 3.1. Ethylene production in dicamba susceptible (dotted line, ○) and resistant (solid line, ●) kochia treated with 280 g ha^{-1} dicamba measured 0, 6, 12, 24, 48 and 72 hours after treatment. Estimated equations for lines are $\hat{y}$ = 7.8(time) + (-45.7) and $\hat{y}$ = 13.5(time) + 71.8 for resistant and susceptible kochia, respectively. Time = hour after treatment.

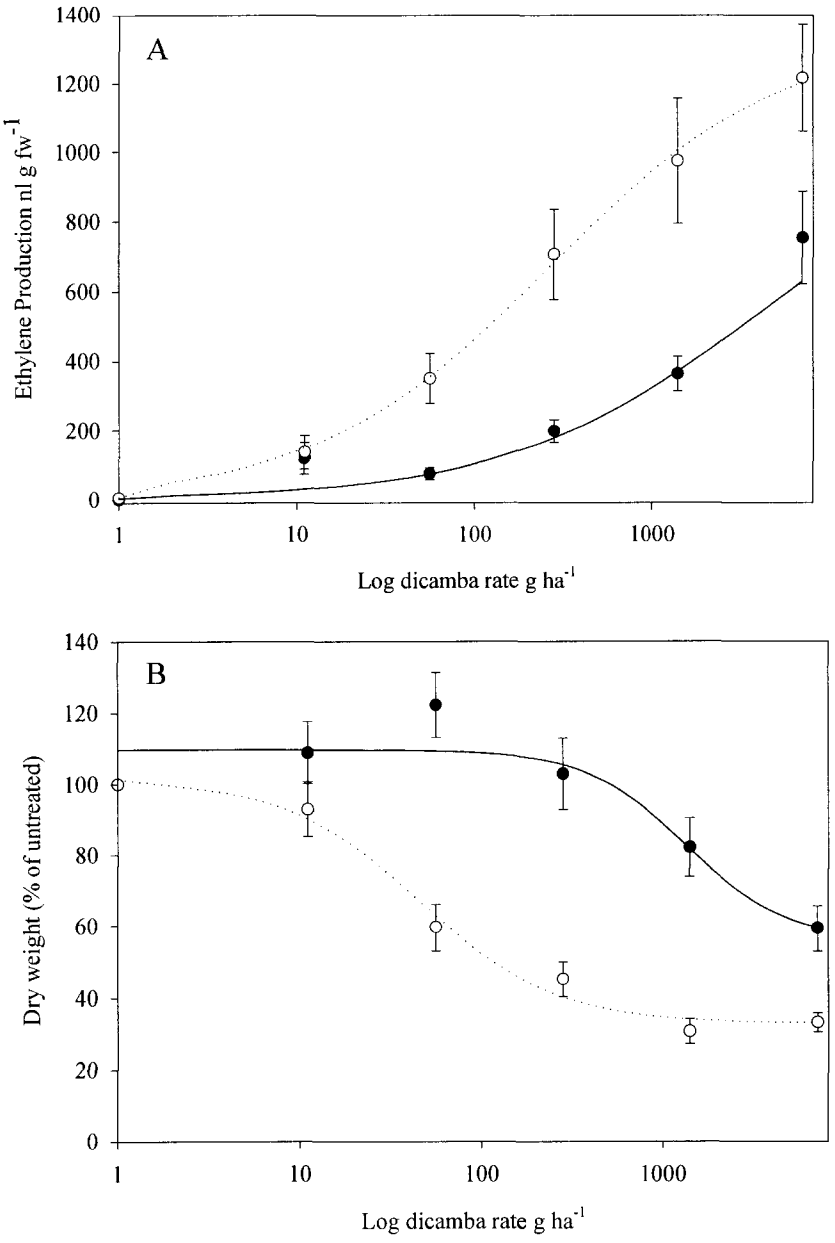

Figure 3.2. The ethylene production (A) and dry weight (B) of dicamba resistant (solid line, ●) and susceptible (dotted line, ○) kochia 48 hours after dicamba applications of 0, 11, 56, 280, 1400, and 7000 g ha^{-1}. Equations for lines (ethylene production) are $\hat{y} = 1.8+ [1095.7 - 1.8 / 1 + (\log(dose) - \log(4318.5))^{-0.6}]$ for and $\hat{y} = 0+ [1346.8 - 0 / 1 + (\log(dose) - \log(271.0))^{-0.7}]$ for resistant and susceptible kochia, respectively. Equations for lines (dry weight) are $\hat{y} = 101.3+ [32.9 - 101.3 / 1 + (\log(dose) - \log(45.2))^{-1.2}]$ for and $\hat{y} = 109.7+ [55.8 - 109.7 / 1 + (\log(dose) - \log(1331.4))^{-1.6}]$ for resistant and susceptible kochia, respectively. Dose = herbicide rate.

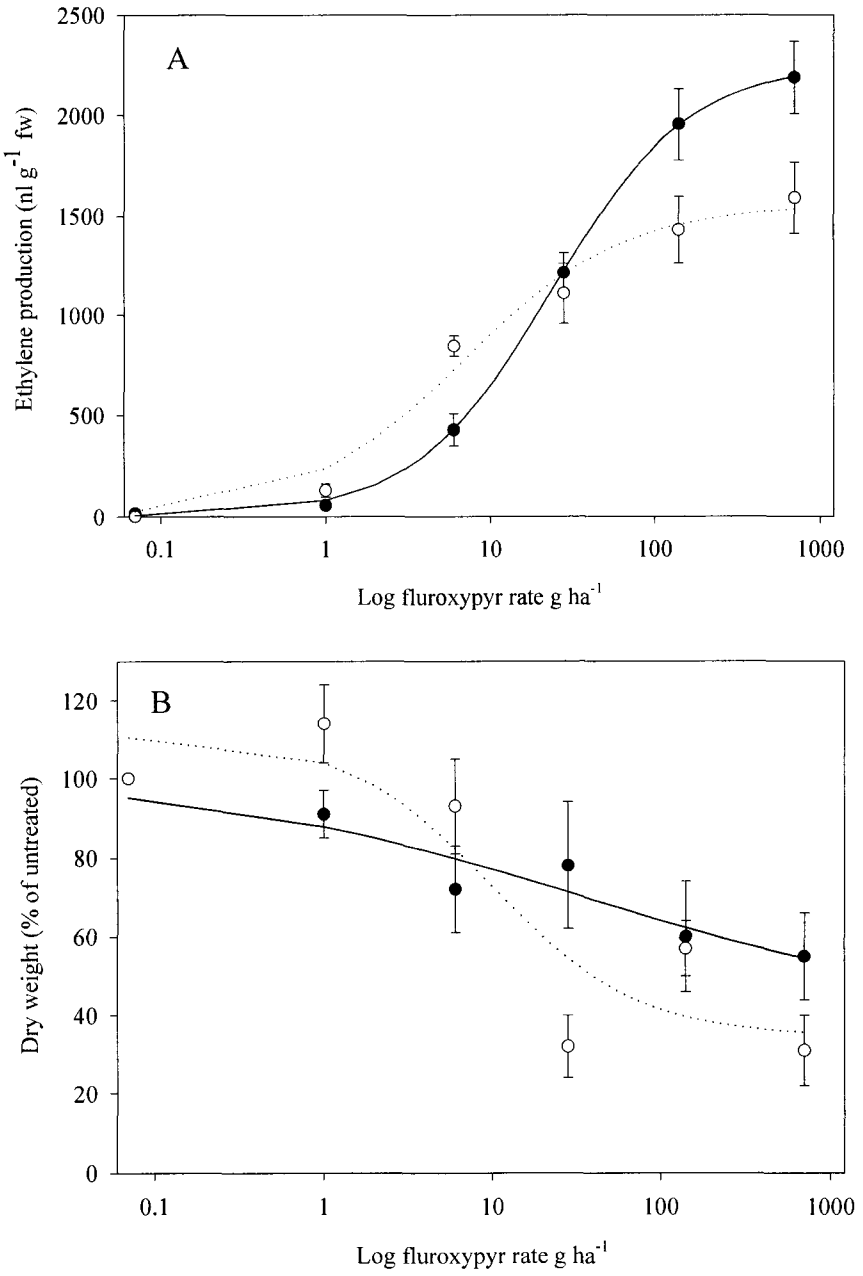

Figure 3.3. The ethylene production (A) and dry weight (B) of dicamba resistant (solid line, ●) and susceptible (dotted line, ○) kochia 48 hours after fluroxypyr applications of 0, 1, 6, 28, 140, and 700 g ha^{-1}. Equations for lines (ethylene production) are $\hat{y} = 0+ [2252.6 - 0 / 1 + (\log(dose) - \log(23.8))^{-1.0}]$ for and $\hat{y} = 0+ [1558.8 - 0 / 1 + (\log(dose) - \log(7.0))^{-0.9}]$ for resistant and susceptible kochia, respectively. Equations for lines (dry weight) are $\hat{y} = 100.4+ [40.0 - 100.4 / 1 + (\log(dose) - \log(33.8))^{-0.4}]$ for and $\hat{y} = 111.0+ [34.6 - 111.0 / 1 + (\log(dose) - \log(9.9))^{-1.0}]$ for resistant and susceptible kochia, respectively. Dose = herbicide rate.

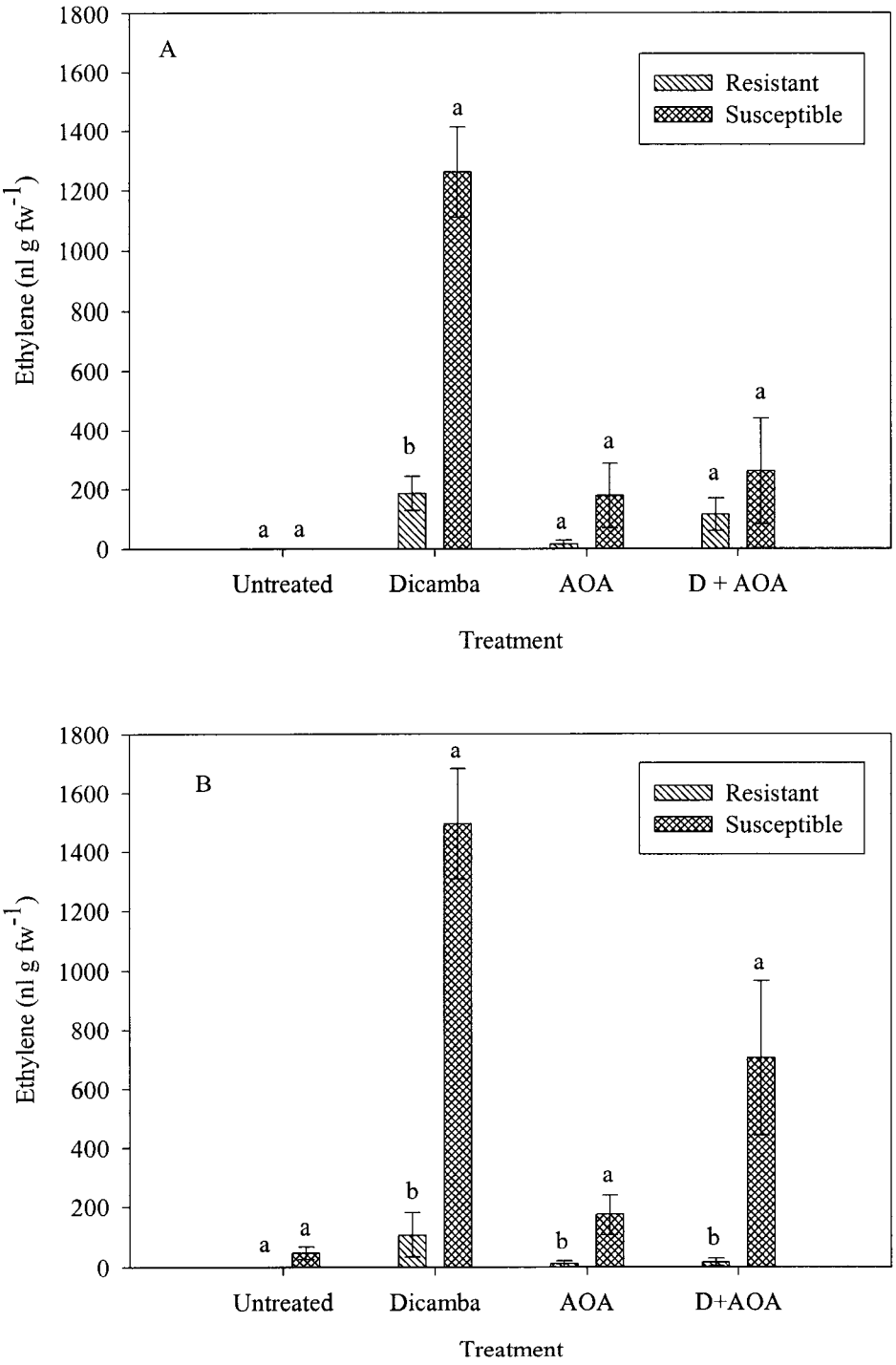

Figure 3.4. The effect of 280 g ha^{-1} dicamba, 100 μM AOA, and 280 g ha^{-1} dicamba plus 100 μM AOA on ethylene production by dicamba susceptible and resistant kochia 48 hours after treatment. Each point is the average of five replications ± SE. Resistant and susceptible means for each treatment are separated by Tukey-Krammer mean separation procedure. Mean separations cannot be compared between treatments. Experiments 1 and 2 are A and B respectively.

Chapter 4

The Inheritance of Dicamba Resistance in Kochia (*Kochia scoparia* L. Schrad.)

David S. Belles*[1], Philip H. Westra[1], Scott J. Nissen[1], Sarah M. Ward[1]

[1]*Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins, CO 80523-1177*

*Corresponding author

Experiments were conducted to determine the inheritance of dicamba resistance in an inbred kochia population. Dicamba resistance in kochia is not absolute with resistant plants showing some epinasty and stunting but no chlorosis or necrosis. Susceptible plants show severe epinasty and chlorosis and die within two weeks. Dicamba susceptible (S-7710) and dicamba resistant (R-9425) plants that had been self pollinated for six and seven generations, respectively, to obtain 99% homozygosity for resistance were crossed (S-7710 x R-9425). Inheritance of resistance was characterized by examining the F_2 segregation pattern. F_2 plants and plants from the parent R and S populations were treated with 280 g ha^{-1} dicamba when they were 2 to 4 cm tall. Fourteen days after treatment an initial examination of the plants revealed the presence of intermediate phenotypes in the F_2 population. Therefore, plants were scored as R (resistance level the same as the treated resistant parent population), S (total plant necrosis), and I (intermediate, more epinasty and stunting than R plants but no tissue necrosis). R, I, and S plants did not segregate consistently in a 1:2:1 ratio in the F_2 generation. A χ^2 homogeneity of ratio test was significant indicating significant variation between F_2 families. Dicamba resistance in kochia is not due to a single nuclear gene and does not consistently fit simple modified hybrid ratios for a two gene system. At least two genes appear to interact to produce the dicamba resistance phenotype observed in this kochia population. F_2 populations did segregate in a 3:1 ratio of non-chlorotic to chlorotic phenotypes indicating the ability of the resistant plants to delay senescence may be under the control of a dominant allele while more genes are involved in the other resistance phenotype characteristics.

Nomenclature: Dicamba, 3,6-dichlor-2-methoxybenzoic acid; kochia, Kochia scoparia L. Schrad.

Key words: Genetics, herbicide resistance, dicamba, auxin, Kochia scoparia.

In 1993, a portion of kochia samples (*Kochia scoparia* L. Schrad.) collected from a field near Morril, NE after many years of annual auxinic herbicide treatment, survived 1120 g ae ha^{-1} dicamba application. Following this discovery, more than 200 kochia samples from Colorado were collected. Most of the samples were susceptible to dicamba. However, 8 populations were injured less than expected from a baseline established from analysis of 59 trials of dicamba on kochia from 1963 to 1994. Therefore, 80 kochia samples were collected from 49 other locations with unexplained response to dicamba as identified by industry representatives from 1996 to 1998. When treated with dicamba at rates of 140, 280 and 560 g ha^{-1} all plants showed some symptoms. The apical meristem of plants from one location died but the axillary meristems produced new branches. There was a continuum of responses ranging from a slight arrest of normal growth to severely chlorotic in plants from the other locations. Fifteen of the 49 locations screened had lower kochia injury than the baseline (Howatt 1999).

Despite the identification of some dicamba tolerance in 15 of 49 kochia populations, or 31%, and the continued use of dicamba, dicamba tolerant kochia has not become a major problem in production agriculture in Colorado. Several factors influence development of herbicide resistant weed populations: selection intensity, generation time, genetic variation in the population, initial mutation frequency, relative ecological fitness of resistant and susceptible weeds, gene flow, response to density dependent and independent factors, and the mode of inheritance (Warwick 1991). Selection intensity is the most important factor governing the development of herbicide resistance. A high selection pressure results in rapid development of an herbicide resistant population as

only those individuals that are resistant survive and produce offspring (Jasieniuk et al. 1996). However, the type of gene that the selection pressure acts upon and its initial frequency in the population will greatly influence how fast a resistance trait develops in the majority of individuals in the population. Most known herbicide resistance is controlled by a single dominant gene (Darmency 1996).

Only a few cases of a recessive allele resulting in an herbicide resistant population are reported. Of 30 herbicide resistant species in one report, seven are due to a recessive gene, 21 are dominant or semidominant, one is additive quantitative, and one is complimentary (Darmency 1994). Green foxtail (*Setaria viridis* L. Beauv.) resistant to trifluralin is one example of herbicide resistance due to a recessive trait. Green foxtail is a highly self fertilized species in which homozygotes are favored (Jasienuik et al. 1994). Recessive alleles are less likely to be selected because the heterozygote is susceptible to herbicide applications. If homozygotes are present and there is continuous selection pressure a resistant population would result.

When dominant alleles confer resistance the trait will quickly be selected for in the population as homozygotes survive and genes are transferred by pollen (Darmency 1994). Polygenic traits that confer resistance are very rare. Of 30 resistance cases listed only one was polygenic (Darmancy 1994). Gene exchange is required that combines favorable alleles into the right genotype to express resistance. Blackgrass, an obligate outcrosser, resistant to chlortoluron is an example of resistance due to two additive genes (Darmancy 1994). However, while technically polygenic this is not truly a quantitative trait due to many genes. Outcrossing and large population sizes are necessary to allow a large number of partial resistance genes to exist in a population.

A monohybrid cross is used to test for the gene type in an herbicide resistant population. In a monohybrid cross individuals from two parent accessions with contrasting forms of the character to study are mated resulting in an F_1 generation. If both parents are homozygous for their respective traits all the individuals in the F_1 generation would be heterozygous. The F_1 generation may be selfed to produce an F_2 generation. If the trait of interest is controlled by a single dominant allele a 3:1 ratio of resistant to susceptible plants would be expected. If the gene is incompletely dominant a 1:2:1 ratio would be expected where the heterozygotes have some resistance but not at the level of the homozygotes. Reciprocal crosses would yield the same results if there was no sex linkage or maternal effects (Klugg and Cummings 1997).

The objective of this study was to determine the number of genes controlling a dicamba herbicide resistance trait in an inbred kochia population. A monohybrid cross between a resistant and a susceptible kochia accession was conducted. Kochia is a diploid and flowers under short days. The flowers are inconspicuous in the upper leaf axils on the plant and contain five sessile stamens per flower. They can be perfect or unisexual (Stubbendick et al. 1997). The resistant accession was assumed to be homozygous for the resistance trait. We hypothesized that the resistance trait in dicamba tolerant kochia was controlled by more than a single gene. Dicamba resistance in kochia has been suspected to be controlled by more than one gene and has been reported as a quantitative trait (Cranston et al. 2001).

Materials and Methods

Kochia Accession Selection.

Resistant kochia seed was collected from population near Henry, NE and susceptible seed was collected from a susceptible population in Montana and sent to Colorado State University. Plants were grown from this seed and screened for dicamba resistance. The survivors were grown and the five most resistant plants to dicamba were grouped together under one large canvas pollination bag to prevent pollen movement from other kochia. These seeds (S_1) were collected, grown, and screened with dicamba. This screening and group pollination process was repeated two more times producing an S_3 population. Ten plants from the S_3 seed were grown. Individual plants were self pollinated by covering each kochia plant with a single glassine pollination bag. The seed produced from each one of these plants was collected. The S_4 seed was planted and grown without selection pressure from dicamba. Two plants from each accession were raised and the S_5 seed was collected. S_5 seed was planted and screened with 140 g ha-1 of dicamba. The largest two plants from each accession were transplanted, raised, and self pollinated two more times to produce an S_7 population. Individual susceptible kochia plants were raised and self pollinated three times without selection after the group pollination procedure to produce and S_6 population. All of the plants were susceptible in the susceptible population when treated with dicamba. S_6 susceptible kochia and S_7 resistant kochia populations were crossed.

Parent Dose-Response Experiments.

The dicamba resistance level of parent accessions was evaluated in a dose response study. Kochia was planted in individual six cell pack flat insert containers[1]. Each individual cell was 3 cm by 3 cm by 4 cm. The cells were filled with dry Metro

Mix 200[2]. Seeds were sprinkled on the surface of the soil and covered with 0.3 cm of dry soil. The flats were then watered until the entire soil profile was wet and afterwards for 90 s 4 times a day. When all the plants emerged they were thinned to one plant per cell. Plants were grown in the greenhouse under a 14/10 h day/night photoperiod supplemented with 400 w sodium halide lights. Day night temperatures were 24 and 18 C, respectively. Plants were sprayed when they were approximately 11 cm tall. Dicamba was applied at 11, 56, 280, 1400, and 7000 g ha^{-1}. An untreated control was also included. Three weeks after treatment, plants were harvested, dried at 60 C for seven days and weighed. Five plants were harvested in each treatment and the experiment was repeated once.

Genetic Crosses.

Parent plants designated as pollen donors were planted approximately one week prior to the seed parents. This allowed them time to flower and produce pollen before the maternal parent's flowers had developed and produced pollen. Plants were planted and grown as in the dose response experiment. Day night temperatures were 24 and 18 C, respectively. Kochia is protogynous and the stigma is receptive before the anthers dehisce. Therefore, pollen was moved from flowering plants to those with protruding receptive stigmas. All of the flowers on the branch or terminal inflorescence were pollinated. Those flowers that were not pollinated were removed. Each branch and terminal inflorescence was covered with dialysis tubing. Eighteen crosses and reciprocal crosses were attempted. The flowers were watched each day and those that developed anthers were emasculated before they could dehisce or the flower was removed. Stigmas

of pollinated flowers often turned brown and died before anthers on the pollinated plant developed increasing our confidence that a cross was successful. In some cases the anthers dehisced before they could be emasculated and these crosses were discarded. A single seed was produced from each flower. Five to ten seeds were collected from each plant.

Testing for Herbicide Resistance.

The F_1 seed from each cross was planted in the same manner as previously described. Five individuals from each cross were self pollinated. F_2 seed was collected from these individuals and approximately 100 to 200 seeds from one or two self pollinated individuals per cross were planted in two 30 cm^2 flats[3]. Seedlings were treated with 280 g ha^{-1} dicamba. All plants were counted before treatment. Fourteen days after treatment susceptible, resistant, and intermediate phenotype plants were counted. At 14 DAT, R and S classes were considered only. However, after further examination it was decided there were at least three classes of response and the plants were recounted 20 DAT considering R, I, and S classes. Susceptible plants were yellow and usually lying on the soil surface. Resistant plants were green, upright and all the leaves were horizontal to the ground. Intermediate plants were green, upright but leaves were usually folded against the main stem. Intermediate plants were also generally shorter than resistant plants. The F_2 screen was repeated once.

Statistical Analysis.

Data from each F_2 screen was analyzed separately. Regression analysis was used to determine the effect of herbicide dose on ethylene production and kochia dry weight.

Dose response residual mean square and predicted sum of squares were examined and a log-logistic non-linear curve fitted to the data for each herbicide (Seedfeldt et al. 1995). Frequencies of R, S, and I plants were tabulated for the F_2 population. Chi-square analysis was used to compare the observed F_2 segregation to specific expected genetic ratios. A chi-square homogeneity of ratio test was conducted to compare the segregation ratios between families (Gomez and Gomez 1984).

Results and Discussion.

Parent Dose-Response Experiments.

The effect of dicamba on the susceptible kochia accession (7710) was 30-fold greater than on the resistant kochia accession (9425). GR_{50} values for resistant kochia were determined as a parameter in the log-logistic model of the reduction in kochia dry weight as a percent of the control (Figure 4.1). GR_{50} values were 1331 and 45 for resistant and susceptible kochia, respectively, an R/S ratio of 30. In general, resistant plants showed less severe epinastic symptoms and no chlorosis or necrosis compared to susceptible plants. The resistance trait in accession 9425 was not complete, however, as some epinastic symptoms did develop. The plants were stunted and these effects were more severe as the dicamba dose increased. The most striking difference between the resistant and susceptible accessions was the ability of the resistant accession to remain green after the susceptible plants had died.

F_2 Segregation.

F_2 plants treated with dicamba at 280 g ha^{-1} showed at least three responses, but the ratio of R, I, and S plants did not fit a 1:2:1 ratio (Table 4.1). Probabilities were less than 0.05 for all of the families in experiment one and six of the eight families in experiment two indicating that the data do not fit a 1:2:1 ratio There was variation in the ratios between F_2 families in each experiment so that the families could not be pooled. Homogeneity of ratio chi-square values among the F_2 families were ($\chi^2 = 45.0$, df = 7, probability = <0.0000) and ($\chi^2 = 96.7$, df = 7, probability = <0.0000) for experiment one and two, respectively.

When screening herbicide resistant weed populations most weed science researchers evaluate the herbicide effect on the plants as "dead" or "alive". An intermediate class is created by researchers to place heterozygote plants showing an intermediate phenotype between the two parents. Therefore, because only one intermediate phenotype is usually used, the probability that the F_2 population will fit a 1:2:1 ratio is increased and single gene inheritance can be incorrectly concluded (Darmency 1994). F_2 families in the second experiment were recounted assuming two intermediate classes (Table 4.2.). Chi-square probabilities exceeded 0.05 for five of eight families suggesting the resistance trait may be controlled by two genes in a 6:3:3:4 ratio; however, it is difficult to explain the observed phenotypes by this segregation ratio. There is not a clear separation between intermediate classes or between resistant and intermediates. For example one intermediate is not epinastic but yellow while the other is yellow but not epinastic. All plants that survive remain green but are in many sizes and show different levels of epinasty that cannot be easily classified into groups. This

observation violates one assumption of Mendelian genetics, that in each case distinct phenotypic classes are produced. The other assumptions are that the genes considered in each cross are unlinked and assort independently, that complete dominance exists, and that all the P_1 crosses involved homozygous individuals so that the F_1 generation will have only heterozygous individuals (Klug and Cummings 1997).

If more than one gene is involved in the inheritance, part of the variation between crosses could be due to heterozygosity in the parent population. When one gene is involved after six generations of self pollination the population will be approximately 90% homozygous. However, if more genes are involved more self pollination events will be required to obtain 90% homozygosity. Therefore, since the kochia was only self pollinated for seven generations only there may still be some heterozygosity in the population that would account for the variation in segregation ratios between crosses.

When only the green non-senescent plants were considered, F_2 populations did segregate in a 3:1 ratio of non-chlorotic to chlorotic phenotypes. This may indicate that the ability of the resistant plants to delay senescence is under the control of a dominant allele while more genes are involved in the other resistance phenotype characteristics (Table 4.3).

Inheritance of most other herbicide resistance traits has been found to be controlled by a single dominant or semi-dominant gene. ALS resistance in kochia (Saari et al. 1994), and common cocklebur (*Xanthium strumarium* L.) (Ohmes and Kendig 1999) was found to be a single dominant gene, 3:1 resistant to susceptible in the F_2 population. Other species with resistance due to a single dominant or incompletely dominant allele include prickley lettuce (*Lactuca serriola* L.) (Mallory-Smith et al. 1990)

and annual sowthistle (Sonchus oleraceus L.) (Boutsalis and Powles 1995), and eastern black nightshade (*Solanum ptycanthum* Dun.) (Volenberg and Stoltenburg 2002). Polygenic inheritance or even resistance due to two genes is rare. Of the 30 cases of resistance where the inheritance was tested and reviewed there were only two instances where resistance was due to two genes, one where it was due to three and none where it was due to more than three genes (Darmency 1994). A large population is required to facilitate necessary gene exchange to combine favorable alleles for herbicide resistance by providing a large number of partial resistance alleles (Darmency 1996). However, with recurrent selection a resistant population can develop with changes in gene frequency at several loci (Gasquez 1997). In order for resistance to be conferred by many genes herbicide selection pressure must be low to allow individuals with only some of the resistance alleles to survive. This may be accomplished if low doses are applied or the herbicidal activity is low (Gasquez 1997).

Sulfonylurea and triazine herbicides are highly target specific, and interfere with a single enzyme and major pathway. In contrast, phenoxy herbicides generally have a lower selection pressure and appear to affect more than a single enzyme or major pathway. They would be likely candidates against which plants might select for a polygenic resistance trait (Jasienuik et al. 1996). However, auxinic herbicide resistance in wild mustard (*Sinapis arvensis* L.), wheat (*Triticum aestivum* L.), and yellow starthistle (*Centaurea solstitialis* L.) is due to a single gene (Jasienuik 1995, Randhama et al. 1987, and Sabba et al. 2003). Resistance was due to a single dominant gene in wild mustard but due to a single recessive gene in yellow starthistle. Therefore, more than one mechanism may be involved in resistance to auxinic herbicides (Preston and Mallory-

Smith 2001). A combination of these mechanisms in one species might occur resulting in a trait due to more than one gene.

A population of resistant kochia with a polygenic resistance trait would increase more slowly than a population with a resistance trait due to a single dominant allele. Heterozygotes of a rare favored dominant allele will express at least part of the phyenotype and will survive, and quickly establish a resistant population (Jasienuik et al. 1996). Quantitative resistance genes can be diluted by immigration of susceptible alleles (Gasquez 1997). If the dicamba resistant trait in kochia was quantitative this could explain why auxinic herbicide resistance has been slow to develop. If the trait is due to two or three alleles and is not truly a quantitative trait the slow development may be due to a low initial frequency of alleles in the natural population. In this study we have shown that the dicamba resistance trait is due to at least two genes, probably three or more.

Sources of Materials

[1]Greenhouse flat inserts, East Jordan Plastics, Inc. P.O. Box 575 East Jordan, MI 49727.

[2] Metro Mix 200, Scotts-Sierra Horticultural Products Co. 14111 Scottslawn Rd., Marysville, OH 43041.

[3]Greenhouse flats, East Jordan Plastics, Inc. P.O. Box 575 East Jordan, MI 49727.

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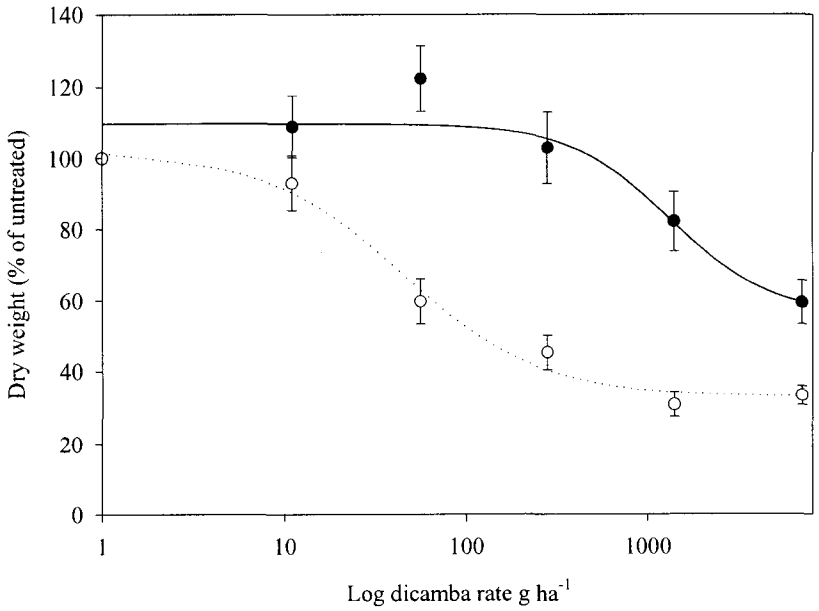

Figure 4.1. Dry weight of dicamba resistant (solid line, ●) and susceptible (dotted line, ○) kochia 48 hours after dicamba applications of 0, 11, 56, 280, 1400, and 7000 g ha^{-1} Equations for lines are $\hat{y} = 101.3 + [32.9 - 101.3 / 1 + (\log(dose) - \log(45.2))^{-1.2}]$ for and $\hat{y} = 109.7 + [55.8 - 109.7 / 1 + (\log(dose) - \log(1331.4))^{-1.6}]$ for resistant and susceptible kochia, respectively. Dose = herbicide rate.

Table 4.1. R, I, and S segregation for kochia dicamba resistance in F_2 families from crosses between dicamba susceptible (7710, pollen receptor) and dicamba resistant (9425, pollen donor) parents and reciprocal crosses (families 9711 and 9750) 20 d after treatment (experiment 1) and 14 days after treatment (experiment 2) with 280 g ha^{-1} dicamba.

Experiment	Family	Phenotype			Total	χ^2 (1:2:1)	Probability
		R	I	S			
		---------- no. plants ----------					
1	7912	105	108	66	279	25.13	0.0000
	7913	104	84	60	248	41.42	0.0000
	7914	68	37	30	135	48.96	0.0000
	7915	120	183	76	379	10.66	0.0048
	7917	68	86	54	208	8.12	0.0173
	7918	113	120	89	322	24.46	0.0000
	9711	55	36	34	125	29.53	0.0000
	9750	54	40	42	136	25.18	0.0000
2	7912	35	111	63	209	8.31	0.0157
	7913	67	74	49	190	12.69	0.0018
	7914	26	72	28	126	2.63	0.2678
	7915	58	136	60	254	1.31	0.5202
	7917	47	93	25	165	8.54	0.0140
	7918	86	67	66	219	36.64	0.0000
	9711	42	33	14	89	23.56	0.0000
	9750	29	99	50	178	7.20	0.0273

Table 4.2. R, I, I, and S segregation for kochia dicamba resistance in F_2 families from crosses between dicamba susceptible (7710, pollen receptor) and dicamba resistant (9425, pollen donor) parents and reciprocal crosses (families 9711 and 9750) and 28 days after treatment (experiment 2) with 280 g ha^{-1} dicamba.

		Phenotype						
Experiment	Family	R	I	I	S	Total	χ^2 (6:3:3:4)	Probability
		---------- no. plants ----------						
2	7912	76	43	28	50	197	3.23	0.3575
	7913	65	32	28	52	177	2.24	0.5243
	7914	42	33	14	33	122	8.44	0.0378
	7915	81	65	33	61	240	13.01	0.0046
	7917	65	44	23	26	158	13.63	0.0035
	7918	67	44	33	56	200	3.24	0.3561
	9711	36	26	13	21	96	5.32	0.1498
	9750	62	35	21	48	166	4.80	0.1874

Table 4.3. R, and S segregation for kochia dicamba resistance in F_2 families from crosses between dicamba susceptible (7710, pollen receptor) and dicamba resistant (9425, pollen donor) parents and reciprocal crosses (families 9711 and 9750) 20 d after treatment (experiment 1) and 14 days after treatment (experiment 2) with 280 g ha^{-1} dicamba.

Experiment	Family	Phenotype		Total	χ^2 (3:1)	Probability
		R	S			
		---------- no. plants ----------				
1	7912	213.0	66	279	0.27	0.6041
	7913	188.0	60	248	0.09	0.7693
	7914	105.0	30	135	0.56	0.4561
	7915	303.0	76	379	4.95	0.0261
	7917	154.0	54	208	0.10	0.7488
	7918	233.0	89	322	1.20	0.2740
	9711	91.0	34	125	0.32	0.5700
	9750	94.0	42	136	2.51	0.1131
2	7912	146.0	63	209	2.95	0.0859
	7913	141.0	49	190	0.06	0.8016
	7914	98.0	28	126	0.52	0.4715
	7915	194.0	60	254	0.26	0.6120
	7917	140.0	25	165	8.54	0.0035
	7918	153.0	66	219	3.08	0.0792
	9711	75.0	14	89	4.08	0.0434
	9750	128.0	50	178	0.91	0.3411

APPENDICES

Appendix A.1.

Characterization of Dicamba Resistance in Collected Accessions

Experimental Procedure.

S_3, S_4, and S_5 kochia seed was planted in 10 by 15 cm pots in Metro Mix 350 potting soil. Five weeks after planting accessions were spayed with 140 g ha^{-1} dicamba when the kochia was 1 to 11 cm tall. There were four replications per treatment. Two weeks after treatment the height and injury of the tallest and shortest plants of each accession in each replication were measured. Herbicide injury was evaluated, resistant and susceptible plants were counted and the percent of plants resistant to dicamba was calculated. Based on the results of this experiment eight kochia accessions were selected as candidates for cross pollination. Susceptible and resistant accessions were chosen for crossing and genetics studies. These lines were treated with 70, 140, 280, and 560 g ha^{-1} dicamba three weeks after planting when the plants were 3 to 8 cm tall to evaluate their dicamba response. Herbicide injury was evaluated one, two, and three weeks after treatment.

Results

Control of S_3, S_4, and S_5 generation kochia ranged from 5 to 100% (Table A.1.1). Control of the same accession in different generations often was not consistent. The resistance trait was still segregating in these accessions creating variation in the resistance

level among individuals within a generation depending on the resistance level of the individual from which seeds were collected in the previous generation. The herbicide application that determined resistance prevented seed production so plants were randomly chosen for self pollination before herbicide was applied. Therefore, a less susceptible plant could have been chosen changing the frequency of resistance in the next generation. A few lines were consistent from generation to generation perhaps because they had lost most of their susceptible alleles or a resistant individual was fortuitously chosen for self pollination. Some of these accessions were chosen as candidates for cross pollination experiments and their dicamba response evaluated at four rates (Figure A.1.1). Accessions 7710, 9428, and 9523 were susceptible to dicamba treatments. The most resistant accession was 9425. Control of 9425 was less than 20 % at 280 g ha^{-1} and was only 50 % at 560 g ha^{-1}. Accessions 9508 and 9426 had an intermediate level of control. Accession 9508 was more resistant than 9426 except at 70 g ha^{-1}. Accessions 9425 and 7710 will be used in future genetic studies because of the large difference in their dicamba response.

Table A.1.1. Number of plants treated, height and injury of the shortest plant, height injury of the tallest plant, overall percent control, number of susceptible plants, number of resistant plants and % of plants that were resistant to 140 g ha-1 dicamba.

Gen.	Acc.	Plants	Height tallest	Height shortest	Injury tallest	Injury shortest	Control	S	R	R
		#	(cm)	(cm)	%	%	%	#	#	%
3	7603	7	9	3	78	91	88	7	0	3
3	7705	4	15	3	13	91	53	2	1	37
3	7710	12	7	2	89	99	95	12	0	0
3	9320	11	12	4	14	96	24	6	6	50
3	9382	14	11	2	53	95	80	11	2	17
3	9397	5	14	3	24	98	59	3	2	39
3	9404	13	10	4	65	88	84	11	2	12
3	9407	7	13	2	26	99	63	6	1	20
3	9426	12	12	4	8	51	29	5	7	61
3	9427	9	7	3	94	100	96	9	0	3
3	9428	7	10	3	53	93	70	5	2	27
3	9433	6	16	4	2	3	5	0	6	100
3	9441	13	8	3	79	100	90	12	0	2
3	9442	10	13	13	11	95	63	7	3	31
3	9508	7	16	3	4	93	45	2	4	65
3	9509	5	12	4	16	58	45	3	2	47
3	9523	8	15	5	33	94	35	3	4	58
3	9425	12	10	2	76	96	88	10	2	15
3	9527	16	10	3	9	100	76	10	6	39
3	93145	4	7	2	79	96	97	4	0	4
4	7603	7	9	3	78	91	88	7	0	3
4	7710	3	13	5	60	85	63	2	1	33
4	9320	0	17	.	5	.	50	0	1	100
4	9404	2	12	5	68	69	66	1	1	67
4	9407	4	16	2	10	55	24	2	2	60
4	9426	1	15	.	46	.	46	0	1	67
4	9428	4	18	9	8	28	28	1	4	93
4	9428	1	11	4	80	90	83	.	.	.
4	9441	1	10	.	78	.	78	1	1	50
4	9442	5	16	9	10	33	8	1	3	79
4	9508	1	26	.	3	.	0	0	1	100
4	9509	1	16	.	58	.	58	1	1	50
4	9425	6	13	6	71	84	50	4	2	39
4	9527	1	21	18	5	.	3	0	2	100
5	7705	1	3	.	99	.	99	1	0	0
5	9382	1	10	.	77	.	80	0	1	67
5	9382	3	8	4	82	88	87	2	1	33
5	9404	1	8	.	70	.	70	1	1	50
5	9407	4	8	5	89	100	91	3	1	28
5	9426	9	9	3	64	94	93	7	2	25
5	9428	4	6	3	78	98	95	4	0	5
5	9508	8	15	4	2	70	18	3	5	65
5	9523	8	9	4	65	99	90	7	1	16
5	9527	1	3	2	47	85	48	1	1	67

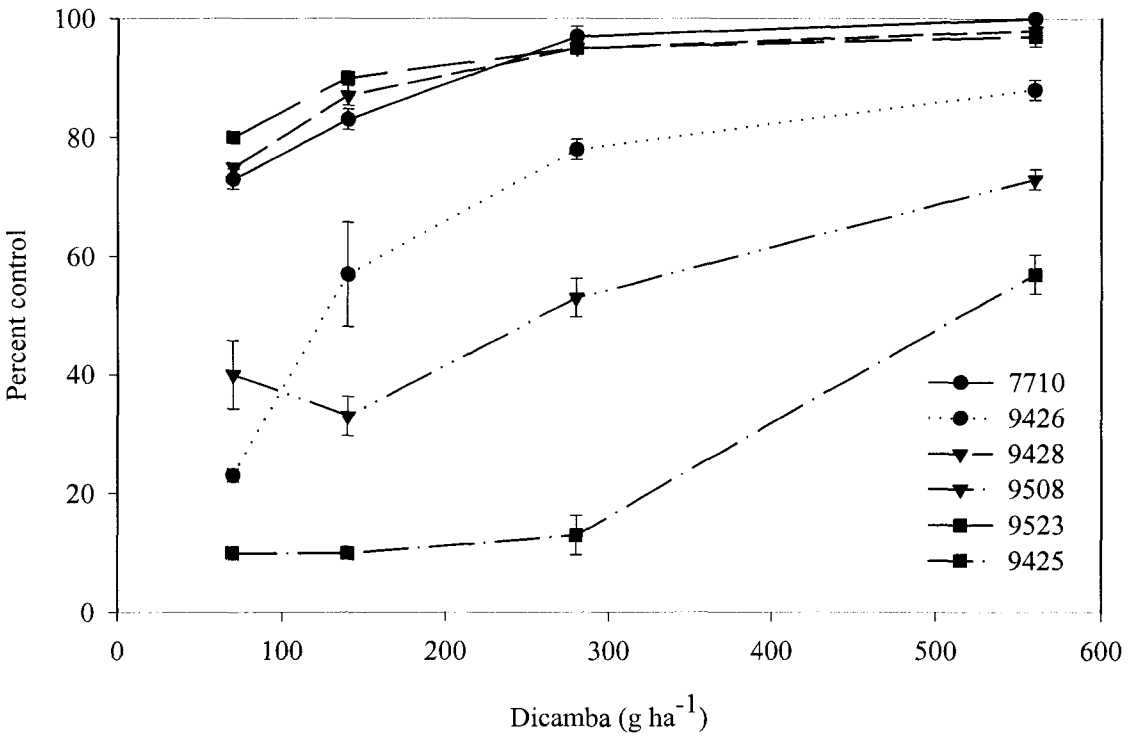

Figure A.1.1. The percent control of six kochia accessions in the greenhouse by 70, 140, 280 and 560 g ha^{-1} dicamba rates three weeks after treatment.

Appendix A.2.

Cross Resistance in Dicamba Resistant Kochia Accessions

Experimental Procedure.

Ten kochia accessions in the S_5 generation were sprayed with 140 g ha^{-1} dicamba, 26 g ha^{-1} chlorsulfuron, and 1400 g ha^{-1} atrazine to test for resistance. Non-ionic surfactant and crop oil concentrate were added to chlorsulfuron and atrazine at 0.5 % v/v and 2.3 L/ha, respectively. Plants were treated when they were 5 to 10 cm tall and injury evaluated 1 and 4 WAT.

Results

Some of the kochia accession collected with a reduced susceptibility to dicamba at 140 g ha^{-1} were also resistant to chlorsulfuron and atrazine (Table A.2.1). Accession 9382 was resistant to atrazine and accession 9426 and 9527 were resistant to chlorsulfuron. All of the accessions tested were less susceptible to dicamba at 140 g ha^{-1}. However, at 280 g ha^{-1} only accessions 9523 and 9425 were resistant to dicamba and these accessions were not resistant to atrazine or chlorsulfuron. If an accession that was susceptible to both atrazine or chlorsulfuron and dicamba could be found then the resistance to the atrazine or chlorsulfuron could be used to identify true F_1 individuals in crosses made to dicamba susceptible and atrazine or chlorsulfuron susceptible parents.

Table A.2.1. S_5 kochia four weeks after treatment with 140 g ha^{-1} dicamba, 26 g ha^{-1} chlorsulfuron, or 1400 g ha^{-1} atrazine.

Accession	Herbicide Treatment[a]			
	Atrazine	Chlorsulfuron	Dicamba	Dicamba[b]
	----------% control----------			
7705	100 a	100 a	5 b	42 b
9382	33 b	100 a	0 c	78 a
9407	100 a	100 a	25 a	70 a
9426	100 a	0 b	0 c	33 b
9428	100 a	100 a	0 c	77 a
9508	100 a	100 a	5 b	34 b
9523	100 a	100 a	25 a	10 c
9425	100 a	100 a	0 c	10 c
9527	100 a	100 a	5 b	80 a

[a]Means followed by the same letter are not different using Tukey-Kramer's adjustment at the 0.05 level of probability.
[b]Dicamba treated at 280 g ha^{-1} and evaluated 2 weeks after treatment.

Appendix A.3.

Testing for Dicamba Resistance in Kochia – Leaf Curvature Assay

Experimental Procedure.

A leaf curvature assay was conducted following a procedure similar to Keller and Volkenburgh (1997). Leaf strips 10 mm by 1.5 mm were cut from the interveinal portion of kochia leaves. One strip was cut from each side of the mid-vein. Leaves were cut from the bottom, middle, and upper sections of the plant. The strips were immediately placed in 5 cm diameter Petrie dishes containing 10 ml of a solution of 10 mM sucrose, 10 mM KCL, 0.5 mM Tris/HCL (pH 6.0). To dicamba treated strips 9.744 ml of the control solution was added and 256 µl of a 1 mM dicamba solution for a final volume of 10 ml with a dicamba concentration of 25 µM. Six leaf strips from a dicamba susceptible or resistant plant were placed in each Petrie plate. There were six replications of the dicamba treated plates and three replications of the untreated plates. The experiment was repeated once.

Results.

There was no difference in the degree of leaf curvature between accessions when untreated but there was a difference when treated with dicamba in both runs of the experiment (Table A.3.1 and A.3.2). Also, in the first run of the experiment there was a difference between treated and untreated susceptible kochia but no difference between treated and untreated resistant kochia. However, in run two there was not a difference

between treated and untreated susceptible kochia. Therefore, although there was a clear difference between accessions in the degree of leaf curvature when treated with dicamba indicating that this may be a useful method to determine dicamba herbicide resistance in kochia, variability in the test may make it unreliable. A difference should consistently be seen between treated and untreated susceptible leaf strips to be confident in the results. The experiment should be pursued to determine the amount of variability in the assay and to try and reduce the variability.

Literature Cited

Keller, C.P., and E. Van Volkenburgh. 1997. Auxin-induced epinasty of tobacco leaf tissues: a nonethylene-mediated response. Plant Physiol. 113:603-610.

Table A.3.1. average leaf curvature in degrees of 36 dicamba resistant leaf strips and 18 susceptible kochia leaf strips measured 24 hours after treatment in two experiments.

Accession	Run 1		Run 2	
	Untreated[a]	Dicamba[a]	Untreated[a]	Dicamba[a]
	--------degree of curvature--------		--------degree of curvature--------	
7710	100 a	173 a	212 a	253 a
9425	83 a	87 b	138 a	120 b

[a]Means followed by the same letter within a run and treatment are not different using Tukey-Kramer's adjustment at the 0.05 level of probability.

Table A.3.2. The average leaf curvature in degrees of 36 dicamba resistant leaf strips and 18 susceptible kochia leaf strips measured 24 hours after treatment in two experiments.

	Run 1[a]		Run 2[a]	
Treatment	7710	9425	7710	9425
	-------- curvature (deg) --------		-------- curvature (deg) --------	
Untreated	100 a	83 a	212 a	138 a
Dicamba	173 b	87 a	253 a	120 a

[a]Means followed by the same letter within a run and treatment are not different using Tukey-Kramer's adjustment at the 0.05 level of probability.

Appendix A.4.

Shoot Gravitropism of Dicamba Resistant and Susceptible Kochia

Introduction.

Auxin plays an important role in shoot gravitropism in plants (Muday 2001). Several auxin resistant genes identified in *Arabidopsis* including aux1 and axr2 have agravitropic roots. The mutant axr2 has agravitropic shoots (Smalle and Van Der Straeten 1997). Several dicamba tolerant kochia lines with agravitropic shoots were observed (Dyer et al. 2001). Shoot agravitropism may be a characteristic of all dicamba tolerant kochia and would suggest possible genes involved in the dicamba tolerance trait.

Experimental Procedure.

The shoot gravitropism of seven kochia accessions was evaluated. Plants were grown until they were approximately 4 to 6 inches tall and then placed horizontal in the dark. After 24 hours the degree the stems had bent was measured. A second test was conducted to compare known dicamba susceptible and dicamba resistant accessions. Four to six inch kochia plants were placed horizontal in the dark. The degree of shoot curvature was measured 1, 2, 3, 4, 5, 6, 7, 8 and 12 h after horizontal placement.

Results.

After 24 h the angle of bending by the shoot of the seven kochia accessions tested were all 90 degrees or greater (Table A.4.1). Although the seven accessions exhibited a variety of responses to dicamba in other studies from resistant to susceptible their shoots

were all sensitive to gravity. Differences in bending did not correspond to the degree of dicamba tolerance observed in previous experiments (Table A.1.1).

When the degree of shoot curvature was monitored 1, 2, 3, 4, 5, 6, 7, 8 and 12 h after horizontal placement the dicamba resistant accession (9425) curved faster than the susceptible accession. After 6 hours there was no difference in the degree of curvature between the resistant and susceptible accession (7710) (Table A.4.2).

Literature Cited

Dyer, W.E., H.J. Cranston, and A.K. Kern. 2001. Physiological characterization of dicamba resistance in kochia. Proc. Western Soc. Weed. Sci. 54:53.

Muday, G.K. 2001. Auxins and tropisms. J. Plant Growth Regul. 20:226-243.

Smalle, J. and D. Van Der Straeten. 1997. Ethylene and vegetative development. Physiologia Plantarum 100:593-605.

Table A.4.1. Shoot angle response of 7 kochia accession to gravitropism as measured by the angle of shoot bending after 24 h in the dark.

Accession	Angle (deg) [a]
9523	128 a
9426	107 ab
9508	107 ab
9425	96 b
7710	94 b
Nunn	92 b
9428	91 b

[a]Means followed by the same letter are not different using Tukey-Kramer's adjustment at the 0.05 level of probability.

Table A.4.2. The shoot bend angle of a dicamba resistant (9425) and susceptible (7710) after 1, 2, 3, 4, 5, 6, 7, 8 and 12 hours after horizontal placement in the dark.

	Hours After Treatment[a]								
Accession	1	2	3	4	5	6	7	8	12
	----------angle (deg)----------								
9425	6 a	30 a	72 a	90 a	90 a	90 a	90 a	90 a	90 a
7710	4 a	24 a	38 b	59 b	81 b	89 a	89 a	89 a	89 a

[a]Means followed by the same letter are not different using Tukey-Kramer's adjustment at the 0.05 level of probability.

Appendix A.5.

DNA Isolation from Kochia

Experimental Procedure.

DNA was isolated from kochia plants using a hexadecyltrimethylammonium bromide (CTAB) extraction buffer. This method yields DNA that is stable for a long time with most PCR inhibitors eliminated and is useful for tough tissues, although it may give a poor DNA yield.

A DNA extraction method for kochia that will be used in future kochia genetic experiments was developed. A homogenization buffer was prepared (100 mM Tris-HCL, (pH 8.0) 1.4 M NaCl, 0.02 M EDTA, 2% CTAB, 0.2% 2-mercaptoethanol) and stored at -4 C. DNA was extracted from kochia by harvesting two young leaves from 4 cm tall kochia plants and placing them in a 1.5 mL microcentrifuge tube to which 500 μL homogenization buffer was added. The tissue was homogenized with a plastic pestle, vortexed and then placed in an incubator at 65 C for 1 h. The tube was centrifuged for 2 min at 13000 rpm 3 times. The supernatant was transferred to a new tube and put on ice. Then 200 μL CTAB buffer was added to the pellet and placed in an incubator at 65 C for 1 h. The sample was then centrifuged at 1400 rpm for 3 min. The supernatant was added to the tubes on ice, centrifuged again, and the supernatant poured into the tubes on ice. To the supernatant 700 μL chloroform and isoamyl alcohol 24:1 was added and the sample vortexed and centrifuged for 15 min at 1400 rpm. Afterwards, 400 μL cold (-20 C) isopropyl alcohol was added and mixed gently. Samples were placed in a refrigerator

at 1 C overnight. Samples were then centrifuged at 1400 rpm for 20 min, the supernatant removed and 700 μL cold 100% ethanol added. The centrifuge step was repeated, the supernatant removed and the pellet allowed to air dry. The dry pellets were re-suspended in 150 μL TE buffer (1 M Tris pH 8.0, 0.5 M Na_2EDTA pH 8.0). DNA quality was checked by running it on a 1% agarose gel and measuring the 260/280 nm absorbance ratio.

Results.

Absorbance ratios (260/280 nm) were 1.29, 1.32, 1.79, and 2.07 for the 4 samples of extracted DNA. A ratio of 1.6 indicates large amounts of nucleic acids relative to protein. A pure preparation should yield between 1.9 and 2.0. Four bands were observed on the agarose gel.

Appendix A.6.

Relative Growth of Dicamba Resistant and Susceptible Kochia Accessions

Experimental Procedure

The relative growth of a dicamba resistant kochia accession (9425) and a susceptible accession (7710) was compared. Four seeds were planted each of 7710 and 9425 accessions in 72 separate 5 by 5 cm pots in Metro Mix 350 potting soil. Seven days after planting, the largest plant from 10 randomly selected cells was harvested. If there was no plant in the selected cell a plant from the next highest numbered cell was harvested. The height, whole plant, shoot, and root weight and leaf area for each plant were measured. New plants were harvested every 7 days. When the plants were 2 weeks old they were transplanted to 10 cm pots. Forty days after planting plants were transplanted to 20 cm pots. A final harvest was made 56 days after planting.

Results.

The total leaf area of the resistant and the susceptible accessions were similar at most of the harvest times even though the resistant accession had larger leaves while the susceptible accession had a greater number of smaller leaves (Figure A.6.1). Only at 56 days after planting was there a difference in the total leaf area of each accession and the resistant plant had the largest total leaf area at this time indicating it would have the greater competitive advantage. The shoot dry weight of the resistant and susceptible accession was also similar until 49 days after planting when the susceptible accession became larger than the resistant accession (Figure A.6.2). The largest difference between

the two accessions was seen in the root dry weight. Thirty five days after planting the resistant accession had significantly less root mass than the susceptible accession and continued to have less root mass through 56 days after planting. At 56 days after planting there was almost a one gram difference in the total root dry weight between the resistant and susceptible accessions (Figure A.6.3).

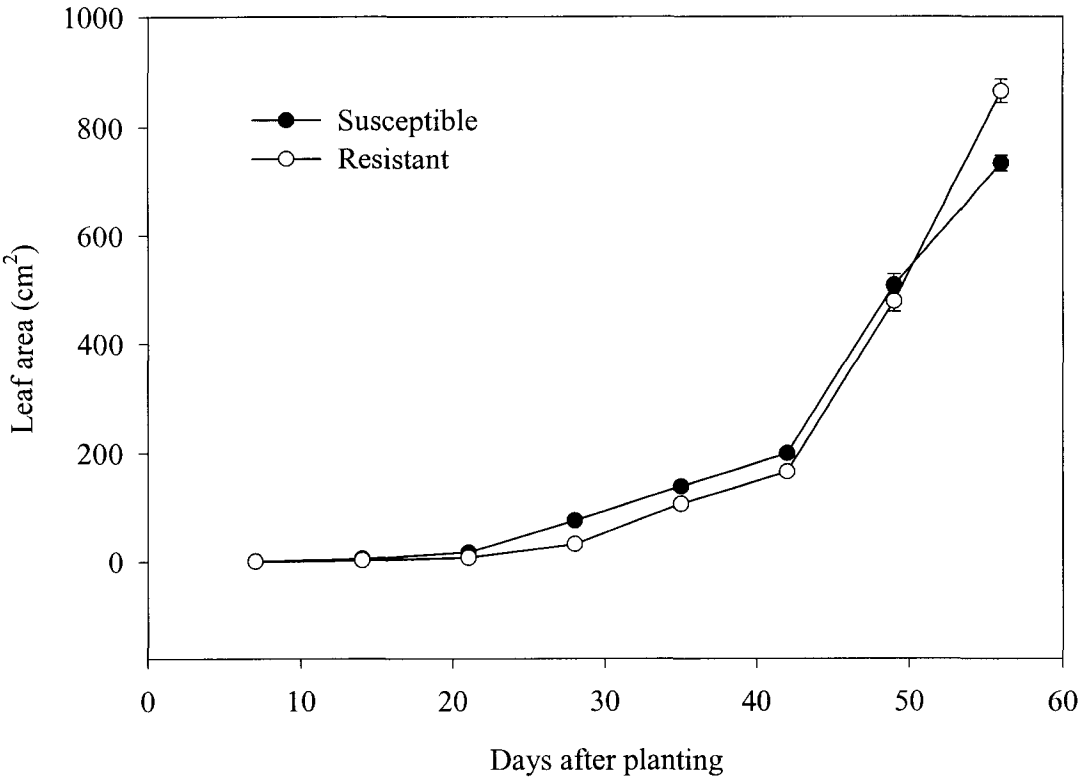

Figure A.6.1. Total plant leaf area of dicamba resistant and susceptible kochia accessions 7, 14, 21, 28, 35, 42, 49, and 56 days after planting.

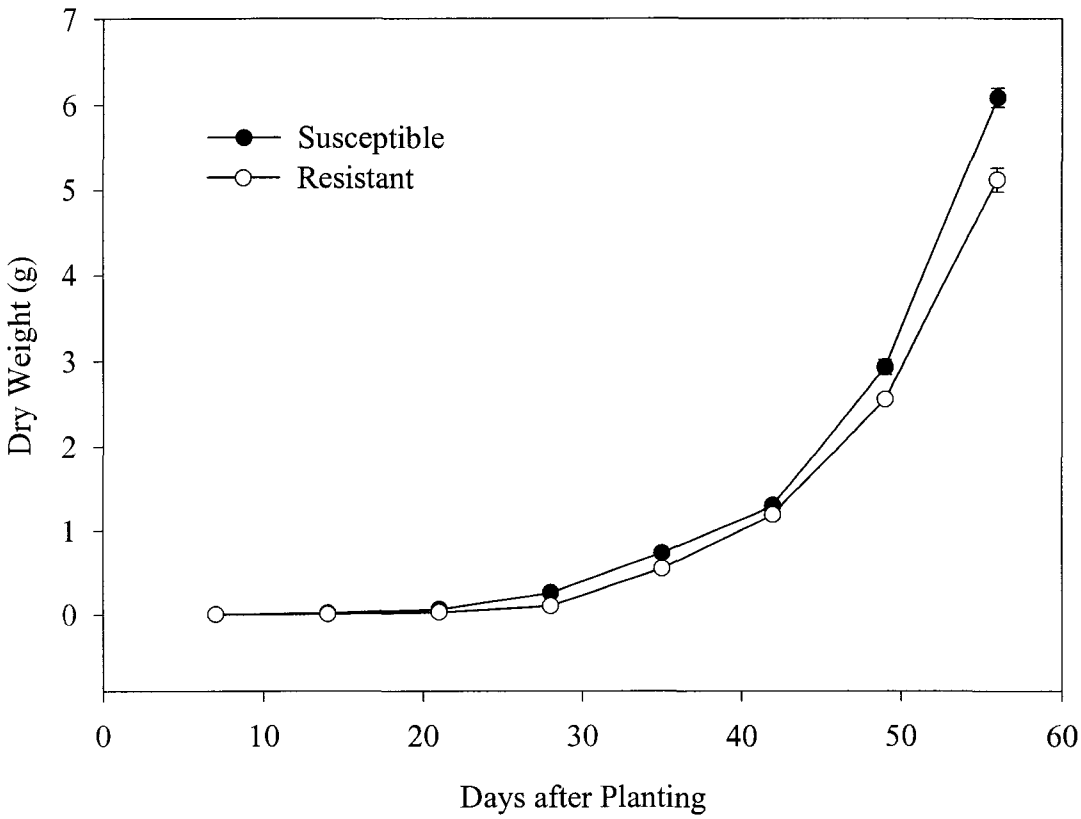

Figure A.6.2. Shoot dry weight of dicamba resistant and susceptible kochia accessions 7, 14, 21, 28, 35, 42, 49, and 56 days after planting.

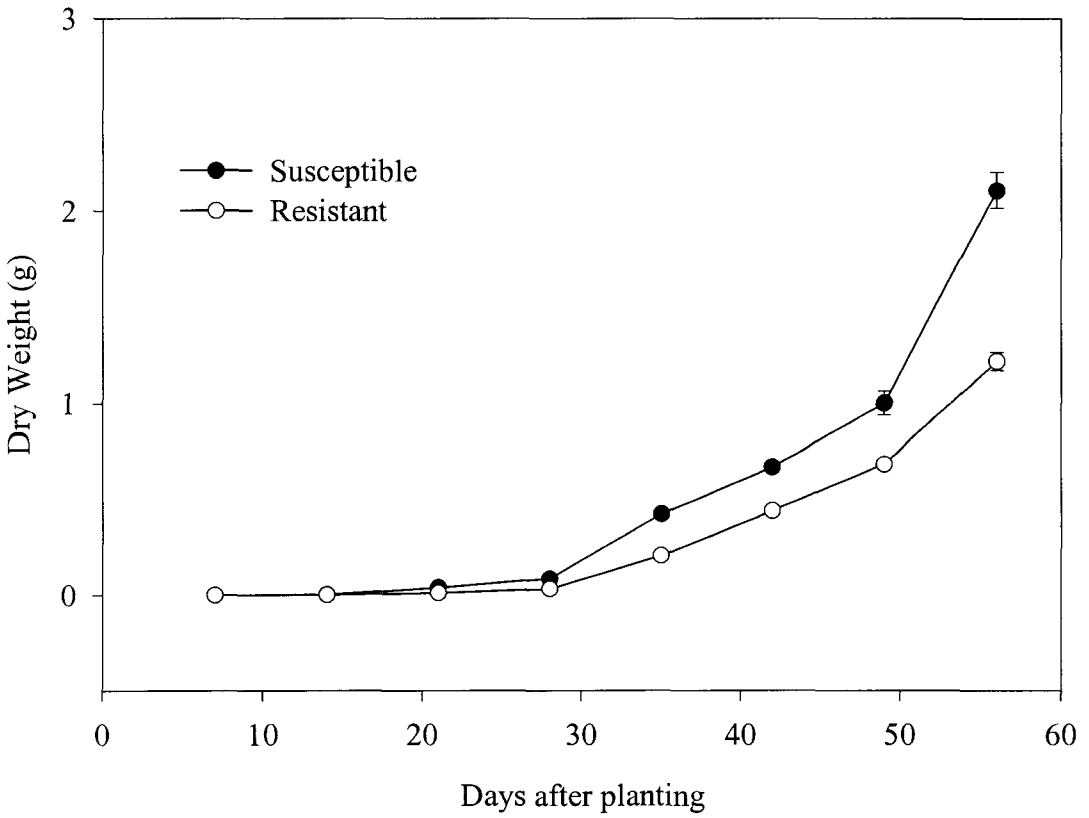

Figure A.6.3. Root dry weight of dicamba resistant and susceptible kochia accessions 7, 14, 21, 28, 35, 42, 49, and 56 days after planting.

Appendix A.7.

Testing for Dicamba Resistance in Kochia – H_2O_2 Production Assay

Experimental Procedure.

The H_2O_2 produced in leaf tissue has been measured during senescence of rice leaves using a colorimetric assay (Chang and Kao 1998). The intensity of the yellow color caused by the reaction of $TiSO_4$ with H_2O_2 was measured at 410 nm. Auxic herbicides such as dicamba have been shown to increase H_2O_2 production in treated plants (Grossman et al. 2001). Dicamba may not induce H_2O_2 production in dicamba resistant kochia while doing so in susceptible kochia and this difference might be detected colorimetrically, providing a novel way to identify auxinic herbicide resistance. Therefore, an experiment was conducted to colorimetrically identify dicamba resistant kochia by H_2O_2 production. A standard curve was developed for H_2O_2 concentrations from 0 to 500 ppm. To test the system, dicamba treated and untreated soybean leaves were used. Into a 96 well micro-titer plate four leaf disks per soybean leaflet were placed in individual wells. Disks from three leaflets from six plants were used. Three plants were treated with a control solution (25 mM $CaCl_2$, 10 mM KCl) and three plants were treated with the 25 μM dicamba added to the control solution. Two rows in the plate were used as positive controls by adding H_2O_2 at concentrations of 10,000, 1000, 500, 250, 125, 63, 31, 16, and 8 ppm to the control solution. Leaf disks were soaked in the treatment solution for 24 h in the dark at 26 C and then frozen at -20 C. After 4 days the disks were removed from the freezer and thawed at room temperature. The leaf discs

were removed from the micro-titer plate, 50 µl 0.1% $TiSO_4$ was added to each well and the color measured by spectrophotometer at 405 nm.

Results.

No difference in color was visually observed between wells with treated and untreated soybean leaf disks as the color change observed was very faint. There was a difference in color as detected at 405 nm by a spectrophotometer (Table A.7.1). However, the change was too faint to make a good colorimetric assay for identification of dicamba tolerant plants (Figure A.7.1). An improved system that produces a greater color change would be useful. The standard curve showed a good color contrast down to 250 ppm H_2O_2 (Table A.7.2, Figure A.7.1). A single leaf disc may not produce sufficient quantities of H_2O_2. More leaf disks per well should be attempted.

Literature Cited

Chang, C. J. and C. H. Kao. 1998. H_2O_2 metabolism during senescence of rice leaves: changes in enzyme activities in light and darkness. Plant Growth Reg. 25:11-15.

Grossmann, K. J. Kwiatkowski, and S. Tresch. 2001. Auxin herbicides induce H_2O_2 overproduction and tissure damage in cleavers (*Galium aparine* L.). J. Exp. Bot. 52(362):1811-1816.

Table A.7.1. OD measurements at 405 nm dicamba treated and untreated soybean leaf discs. Each row contains measurements from disks of a single leaf on a single plant, 4 discs from each of the 3 leaflets.

Treatment	Leaflet 1			Leaflet 2			Leaflet 3		AVG				SE	
(-) dicamba	0.035	0.038	0.048	0.058	0.039	0.043	0.039	0.05	0.039	0.042	0.037	0.044	0.043	0.002
(-) dicamba	0.067	0.052	0.04	0.051	0.048	0.043	0.049	0.059	0.043	0.045	0.052	0.045	0.050	0.002
(-) dicamba	0.062	0.073	0.064	0.058	0.062	0.058	0.072	0.06	0.05	0.057	0.056	0.068	0.062	0.002
(+) dicamba	0.067	0.06	0.061	0.06	0.067	0.072	0.077	0.069	0.067	0.061	0.063	0.059	0.065	0.002
(+) dicamba	0.1	0.091	0.081	0.08	0.075	0.079	0.079	0.071	0.077	0.066	0.07	0.078	0.079	0.003
(+) dicamba	0.075	0.088	0.079	0.081	0.069	0.08	0.077	0.08	0.093	0.113	0.083	0.087	0.084	0.003

Table A.7.2. OD measurements at 405 nm from H_2O_2 standard, and dicamba treated and untreated soybean leaf discs. Each row contains measurements from disks of a single leaf on a single plant, 4 discs from each of the 3 leaflets.

H_2O_2concentration	10000	1000	500	250	125	62	31	16	8	0 ppm
H_2O_2 standard	0.865	0.813	0.434	0.224	0.068	0.05	0.023	0.016	0.009	0
H_2O_2 standard	0.908	0.812	0.391	0.156	0.057	0.03	0.018	0.01	0.004	0

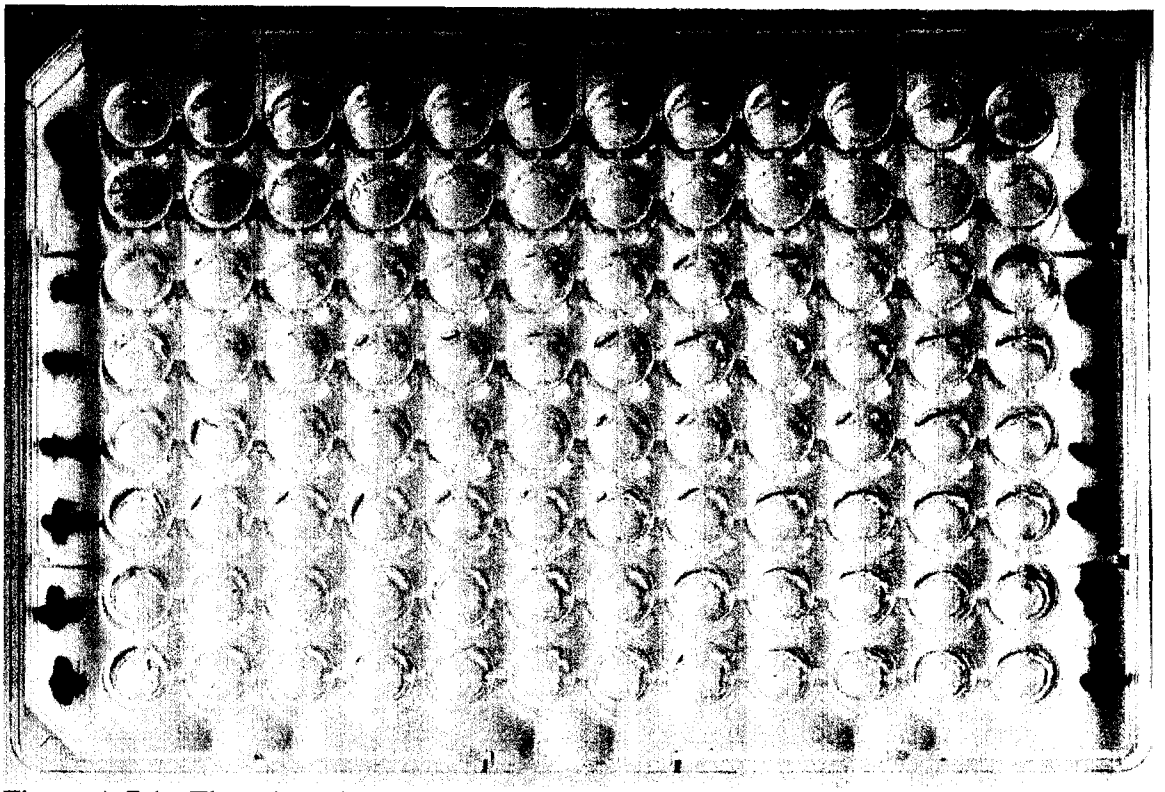

Figure A.7.1. The micro titer plate showing the color produced by $TiSO_4$ when reacted with H_2O_2 produced by untreated and dicamba treated soybean leaf discs, rows 3 through 6. Rows one and two are H_2O_2 standard concentrations of 1000, 500, 250, 125, 63, 31, 16, and 8 ppm.

Appendix A.8.

Growing Kochia In-vitro

Experimental Procedure.

Growth of kochia on sterile media is a useful procedure for conducting many experiments where the effects of compounds added to the media are studied. Herbicides, hormones, and hormone inhibitors may be added to the media, absorbed by the plant roots and their effect studied. A protocol was developed for growing kochia on sterile, solid and liquid media.

Kochia seeds were germinated on a solid media. To make 200 ml of solid media 1.4 g (7g/L) Murashigi and Skoog salt and vitamin mixture and 7.5 g (37.5 g/L) sucrose was added to 200 ml de-ionized water. The pH was adjusted to 5.8 and micro-waved for 1.5 min. Agar at 8 g/L or 1.6 g was added and the mixture boiled in the microwave. The mixture was then autoclaved for 30 min. Kochia seeds were sterilized by abrading them to remove the winged calyx that was a source of fungal contamination and soaking them in a 20% bleach solution for 30 min. Afterwards, they were rinsed three times with autoclaved water. Under a sterile laminar flow hood the seeds were transferred to Petrie plates containing solid media using flame sterilized forceps and autoclaved glassware. The plates were placed in an incubator where the kochia seeds germinated.

After the seeds had germinated they were transferred to sterile test tubes or Erlenmeyer flasks containing liquid media made by the same procedure as the solid media but without agar. Normally, 35 ml test tubes with 5 ml liquid media were used to grow kochia in my experiments. Tubes were capped with autoclaved rubber septa.

Sterilized or filtered compounds could be added to the liquid media through the rubber septum without uncapping the test tube.

Appendix A.9.

Arabidopsis Tolerance to Dicamba, Picloram, Fluroxypyr, and 2,4-D

Introduction.

Arabidopsis thaliana mutants can be used to determine if a particular gene contributes to an observed phenotype. The change in the phenotype of the mutant compared to the wild type demonstrates what the function of that individual gene is. Arabidopsis lines are available with mutations of a single gene or a combination of genes and are available for most of the genes in the Arabidopsis genome. There are many Arabidopsis lines available with mutations in the auxin pathway. These include lines with mutations that confer resistance to auxin. Mutants axr4-1, axr1-3, and aux1-7, have unique resistance phenotypes to auxin. The axr4-1 mutation confers auxin resistance, irregular rosette leaves, defective root gravitropism, and a reduced number of lateral roots. The axr1-3 mutation confers irregular rosette leaves, short petioles, a slightly reduced plant height, an increased number of lateral branches, and auxin resistance. The aux1-7 mutation confers auxin and ethylene resistance, a slight increase in root elongation, and an altered geotropic response. All of the mutants are inherited recessively (Arabidopsis Information Resource 2003). The AXR gene encodes a protein related to the amino-terminal half of the ubiquitin-activating enzyme E1. *Arabidopsis* mutants axr1-12 and axr2-1 reduce ethylene production by reducing expression (greater than 10 fold) of the ACS4 gene that encodes 1-aminocyclopropane-1-carboxylate (ACC) synthase, the enzyme involved in the rate limiting step of ethylene synthesis. Aux1-7

reduced gene expression 1.5 fold but auxin induction of the ACS4 gene was retained (Abel et al. 1995). AUX1 mediates the transport of an amino acid like signaling molecule. Tryptophan is structurally similar to indole-3-acetic acid, a major form of auxin in plants. Therefore, auxin is the likely substrate for the AUX1 gene product (Bennett et al. 1996). The AXR genes play a role in ethylene production while the AUX genes play a much lesser role or no significant role.

Experimental Procedure.

An experiment was conducted to determine the response of wild type and auxin mutant *Arabidopsis thaliana* to direct spray of auxinic herbicides. First the sensitivity of wild type *Arabidopsis* variety Colombia-0 (Col-0) was determined. Col-0 seeds were planted in 12 by 8 by 5.5 cm pots filled with Metro Mix 350 potting soil. After the *Arabidopsis* had emerged they were thinned so that there were approximately 10 plants per pot. When the plants were four weeks old 2,4-D ester, dicamba, and fluroxypyr were applied. 2,4-D ester was applied at 533 g ha^{-1}, dicamba at 280 g ha^{-1}, and fluroxypyr at 140 g ha^{-1} and 1/2, 1/4, 1/8, 1/16, 1/32,1/64, and 1/128 of those rates. An untreated control was also included. Visual evaluations were made 14 days after treatment. The experiment was repeated once. Regression analysis was used to determine the effect of herbicide dose on ethylene production and kochia dry weight. Dose response residual mean square and predicted sum of squares were examined and a log-logistic non-linear curve was fitted to the data for each herbicide (Seedfeldt et al. 1995).

The *Arabidopsis* mutants aux1-7, axr1-3, and axr4-1 were treated with 280 g ha^{-1} dicamba, 140 g ha^{-1} fluroxypyr, and 533 g ha^{-1} 2,4-D and compared to Col-0 wild type

plants spayed with the same herbicides and rates. Four replications were included in the experiment and the experiment was repeated once. Plants were treated when they were 1 to 5 cm in diameter and had 6 to 10 leaves. Visual evaluations were made 2 weeks after treatment and plant biomass was harvested 4 weeks after treatment dried at 60 C and weighed.

Results.

There were significant interactions between experiment, herbicide and rate so the two runs of the experiment were analyzed separately. In experiment one and two, 2,4-D injured *Arabidopsis* the most across all rates applied. Dicamba and fluroxypyr injured *Arabidopsis* similarly in experiment one but dicamba was more injurious in experiment two. The highest rate was required to cause death of the *Arabidopsis* with 2,4-D two weeks after treatment. Fluroxypyr and dicamba did not cause complete death two weeks after treatment even at the highest rate but plants were severely injured (Figures A.9.6 and A.9.7).

Based on the results of the dose response study 533 g ha^{-1} 2,4-D, 280 g ha^{-1} dicamba, and 140 g ha^{-1} fluroxypyr was applied to Arabidopis auxin mutants aux1-7, axr1-3, and axr4-1 and wild type Col-0. The results were analyzed in SAS and because of the presence of significant experiment by herbicide and variety interactions the two experiments were analyzed separately. 2,4-D controlled all *Arabidopsis* varieties better than dicamba or fluroxypyr and control of all varieties by 2,4-D was above 80 percent in both experiments (Figure A.9.8 and A.9.9). Dicamba control of axr1-3 was significantly less than control of other accessions in both experiments. Fluroxypyr control of axr1-3 was also less by fluroxypyr in both experiments. 2,4-D control was less in experiment

two but the difference was not as great as the difference in dicamba control. The other accessions were controlled similarly by all herbicides. Biomass data for both experiments was also analyzed separately. In experiment one accessions Col-0 and axr1-3 were more vigorous than the other accessions when untreated (Figure A.9.10). There was no difference in biomass between accessions for any of the herbicides in experiment one. In experiment two axr4-1 was more vigorous (Figure A.9.11). Within each herbicide treatment axr1-3 produced more biomass than the other mutants when sprayed with 2,4-D, dicamba and fluroxypyr. There was no difference between the other accessions. Axr1-3 had the most resistance to the auxinic herbicides tested. Therefore, the AXR gene may be more important for auxinic herbicide resistance than the mutant genes in the other mutants tested.

Literature Cited

Abel, S., M.D. Nguyen, W. Chow, and A. Theologis. 1995. ASC4, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in *Arabidopsis thaliana*. J. Biol. Chem. 270(32):19093-19099.

Bennett, M.J., A. Marchant, H.G. Green, S.T. May, S.P. Ward, P.A. Millner, A.R. Walker, B. Schulz, and K.A. Feldmann. 1996. Arabidopsis AUX1 gene: A permease-like regulator of root gravitropism. Science. 273:948-950.

The Arabidopsis Information Resource (TAIR), www.arabidopsis.org/servlets/SeedSearcher?action=detail&stock-number=CS8018 and (CS3074, CS3075) on www.arabidopsis.org, Jan 6, 2003.

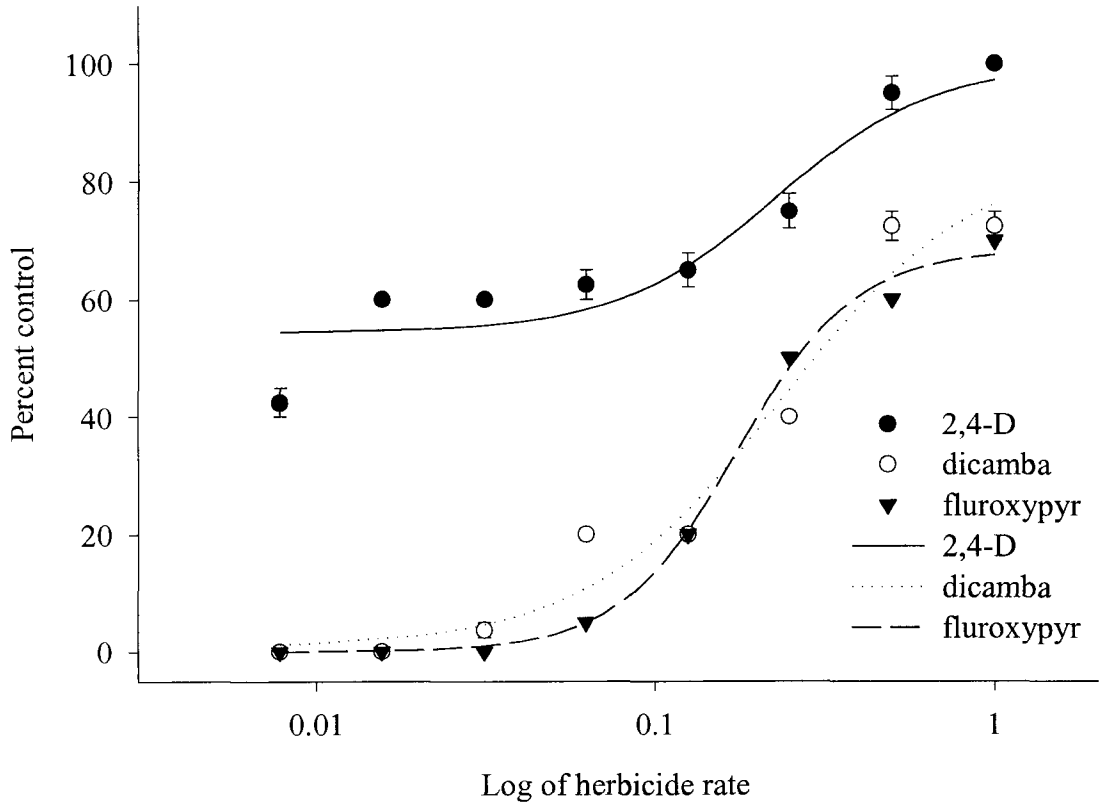

Figure A.9.1. The percent control of Col-0 Arabidopsis 2 weeks after 2,4-D, dicamba, and fluroxypyr applications of 1 , 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and 1/128 the rates of 533, 280, and 140 g ha^{-1} 2,4-D, dicamba, and fluroxypyr, respectively. Equations for lines are $\hat{y} = 54.4 + [100 - 54.4 / 1 + (\log(dose) - \log(.2273))^{-1.8}]$ (2,4-D), $\hat{y} = .8 + [85.1 - .8 / 1 + (\log(dose) - \log(.2365))^{-1.5}]$ (dicamba) and $\hat{y} = 0 + [68.5 - 0 / 1 + (\log(dose) - \log(.1756))^{-2.5}]$ (fluroxypyr). Dose = herbicide rate. Experiment 1.

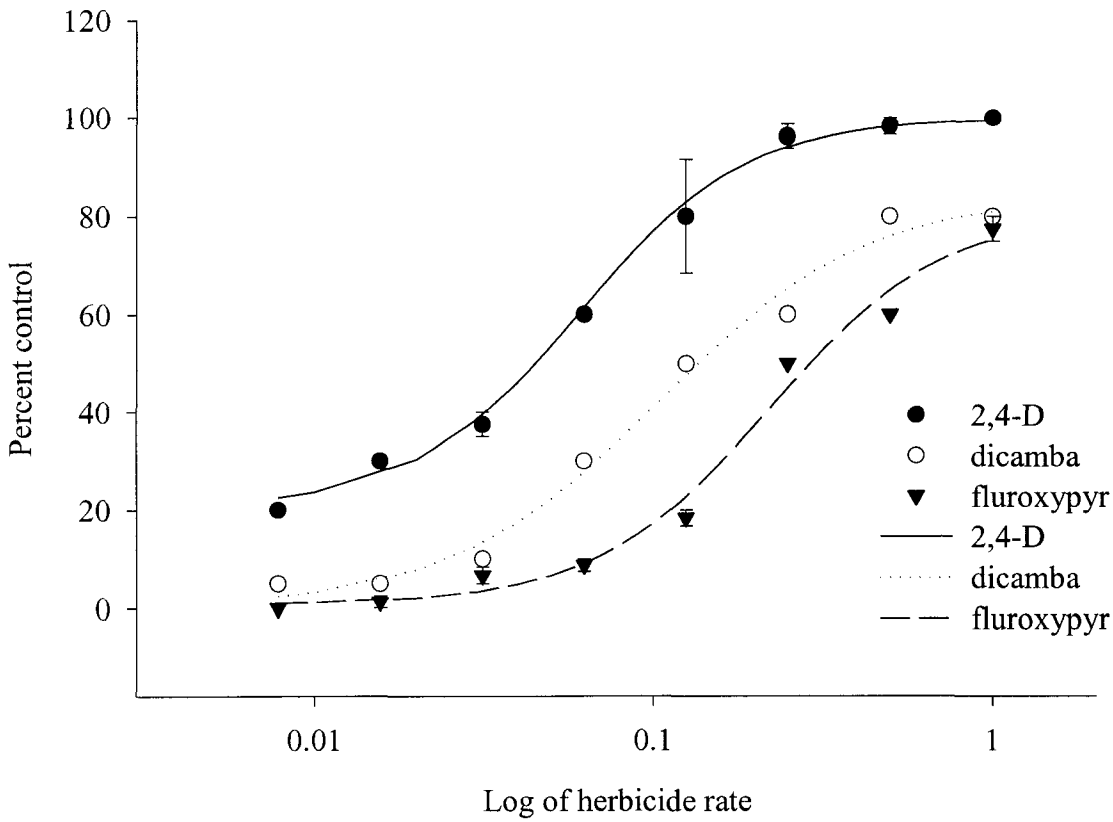

Figure A.9.2. The percent control of Col-0 Arabidopsis 2 weeks after 2,4-D, dicamba, and fluroxypyr applications of 1 , 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and 1/128 the rates of 533, 280, and 140 g ha^{-1} 2,4-D, dicamba, and fluroxypyr, respectively. Equations for lines are $\hat{y} = 20.5 + [100 - 20.5 / 1 + (\log(dose) - \log(.0630))^{-1.8}]$ (2,4-D), $\hat{y} = .3 + [84.3 - .3 / 1 + (\log(dose) - \log(.1042))^{-1.4}]$ (dicamba) and $\hat{y} = .7 + [81.2 - .7 / 1 + (\log(dose) - \log(.2226))^{-1.7}]$ (fluroxypyr). Dose = herbicide rate. Experiment 2.

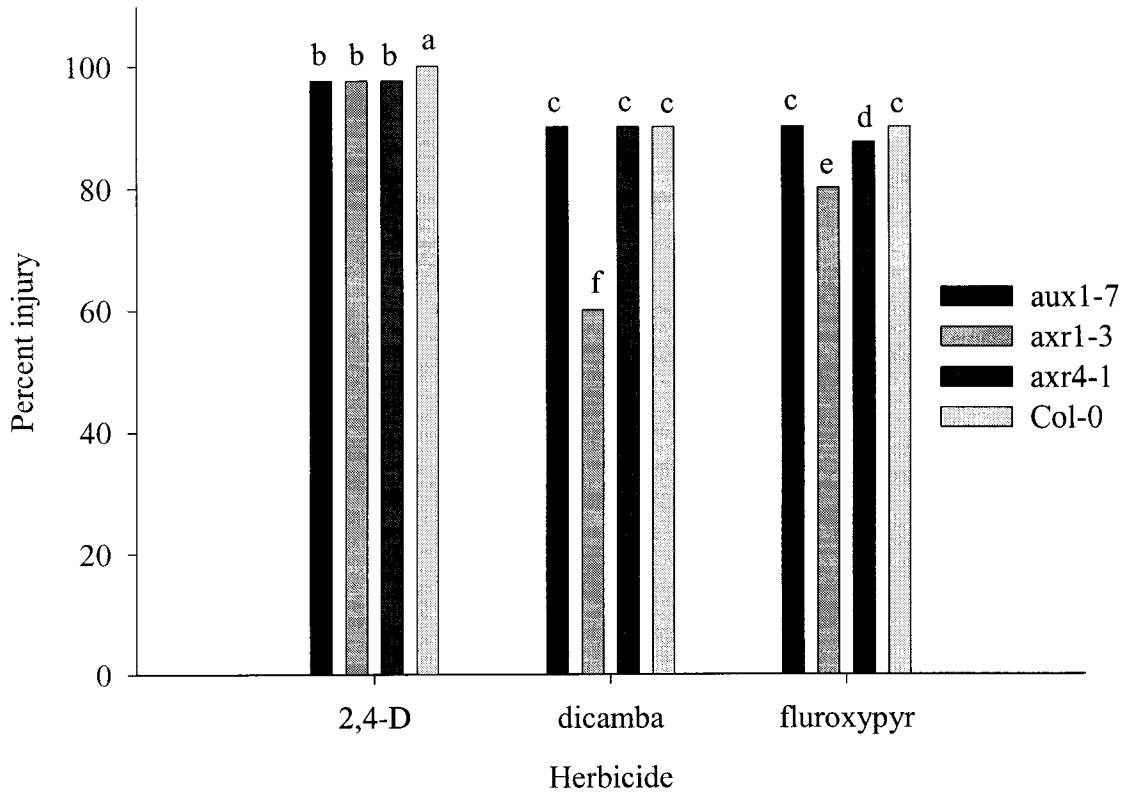

Figure A.9.3. Percent injury of 2,4-D, dicamba, fluroxypyr on Arabidopsis mutants aux1-7, axr1-3, axr4-1, and wild type Col-0 at 533 g ha^{-1}, 280 g ha^{-1}, and 140 g ha^{-1}, respectively, two weeks after treatment. Experiment 1.

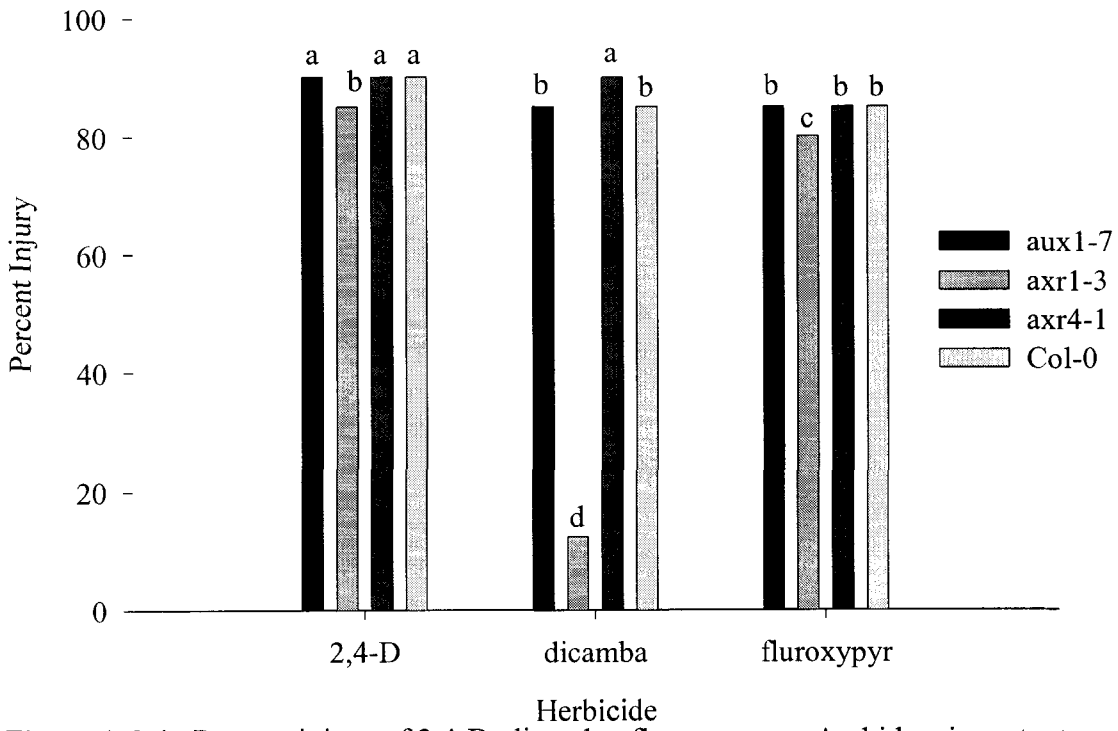

Figure A.9.4. Percent injury of 2,4-D, dicamba, fluroxypyr on Arabidopsis mutants aux1-7, axr1-3, axr4-1, and wild type Col-0 at 533 g ha^{-1}, 280 g ha^{-1}, and 140 g ha^{-1}, respectively, two weeks after treatment. Experiment 2.

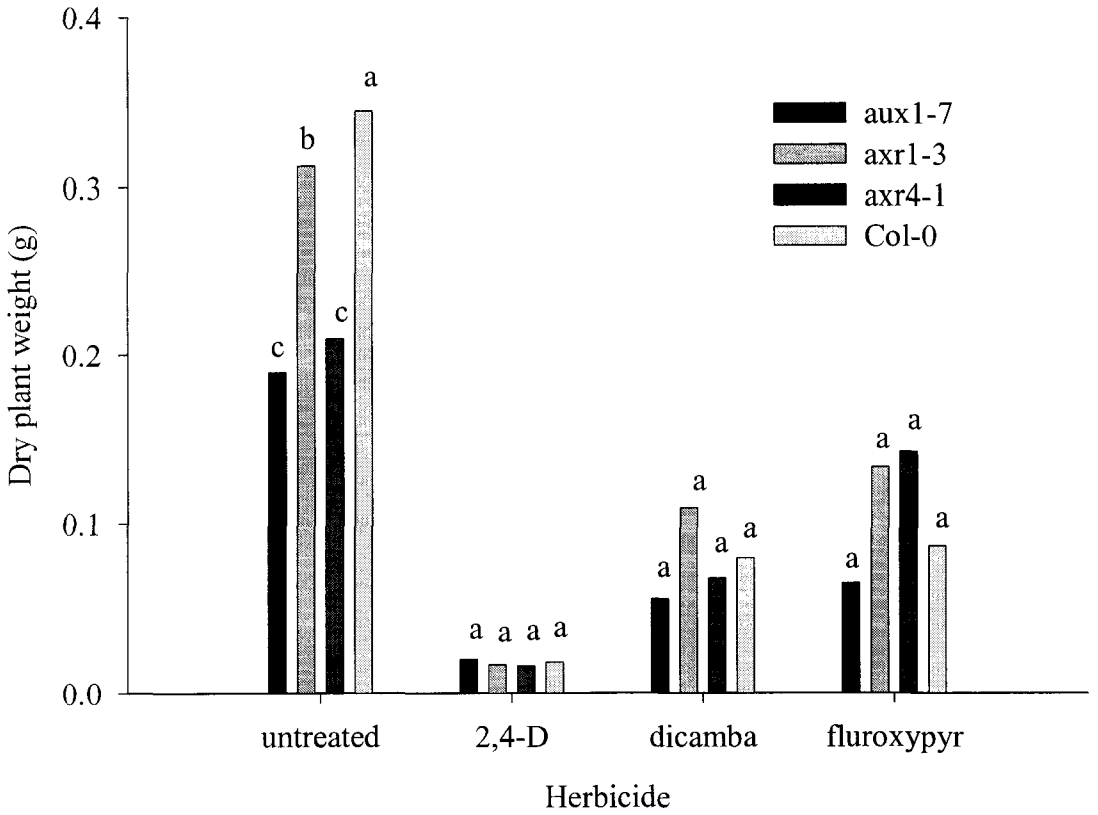

Figure A.9.5. Plant dry weight of Arabidopsis mutants aux1-7, axr1-3, axr4-1, and wild type Col-0 four weeks after treatment with 2,4-D, dicamba, fluroxypyr on at 533 g ha^{-1}, 280 g ha^{-1}, and 140 g ha^{-1}, respectively. Experiment 1.

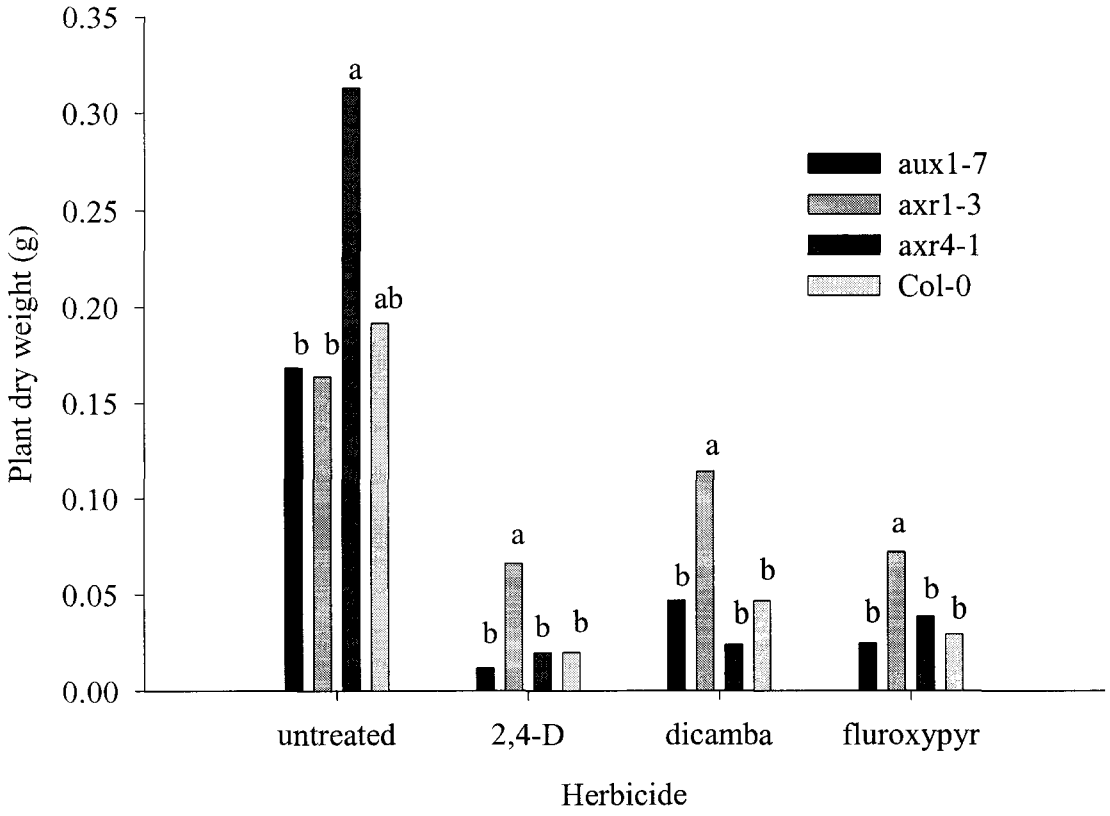

Figure A.9.6. Plant dry weight of Arabidopsis mutants aux1-7, axr1-3, axr4-1, and wild type Col-0 four weeks after treatment with 2,4-D, dicamba, fluroxypyr on at 533 g ha^{-1}, 280 g ha^{-1}, and 140 g ha^{-1}, respectively. Experiment 2.

Appendix A.10.

1999 Kochia Biomass Data from the Response of Four Kochia Accessions to Dicamba and Fluroxypyr

Table A.10.1. The effect dicamba on small kochia biomass in 1999 at ARDEC.

Herbicide Treatment[1]	Rate g ae A^{-1}	Henry	Forsyth	Sato	9426	7710
		----------(g $plant^{-1}$)----------				
Control	0	97 a	115 a	159 a	74 a	218 a
Dicamba	20	30 b	118 a	86 ab	40 a	37 b
Dicamba	39	52 ab	65 a	27 b	32 a	47 b
Dicamba	79	44 ab	119 a	20 b	18 a	15 b
Dicamba	157	73 ab	41 a	38 b	42 a	27 b
Dicamba	314	34 b	63 a	21 b	21 a	26 b
Dicamba	471	83 ab	49 a	25 b	32 a	31 b
LSD (0.05)		62	102	83	61	137

[1]Sunit II a methylated seed oil was added at 1% v/v to each herbicide treatment.

Table A.10.2. The effect of fluroxypyr a on small kochia biomass in 1999 at ARDEC.

Herbicide Treatment[1]	Rate g ae A^{-1}	Henry	Forsyth	Sato	9426	7710
		----------(g $plant^{-1}$)----------				
Control	0	97 a	115 a	159 a	74 a	218
Fluroxypyr	20	62 ab	73 b	40 b	23 a	25 a
Fluroxypyr	39	45 ab	63 bc	85 ab	30 a	64 b
Fluroxypyr	79	29 b	56 bc	24 b	41 a	45 b
Fluroxypyr	157	63 ab	30 c	14 b	21 a	73 b
Fluroxypyr	314	44 ab	38 bc	14 b	42 a	10 b
Fluroxypyr	471	34 b	43 bc	9 b	24 a	21 b
LSD (0.05)		61	41	100	59	136

[1]Sunit II a methylated seed oil was added at 1% v/v to each herbicide treatment.