Characteristics of differential herbicide response in sulfonylurea-resistant Kochia scoparia accessions by Kailayapillai Sivakumaran

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Agronomy
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Abstract:
Three chlorsulfuron-resistant kochia accessions were tested for levels of resistance to sulfonylurea and imidazolinone herbicides, based on whole plant response and sensitivity of the target enzyme. The Minot and Chester accessions were completely resistant to 175 g ha^-1 chlorsulfuron, and I60 values for the Chester accession ranged from 22-fold (metsulfuron) to 196-fold (chlorsulfuron) higher than the susceptible Bozeman accession. However, the Chester accession was 1.5- to 2-fold more resistant to five other sulfonylurea herbicides than Minot, as determined by ALS I60 values. Levels of cross-resistance to four imidazolinone herbicides did not differ appreciably between the Minot and Chester accessions. The intermediate Power accession displayed only 2-to 5-fold increase in resistance over susceptible Bozeman accession to all sulfonylurea herbicides tested, and was not cross-resistant to imidazolinone herbicides.

The highly variable degrees of ALS resistance and cross-resistance among kochia populations may be due to different mutations in the genes encoding ALS. Putative clones have been isolated to determine the molecular basis for the variable responses. DNA sequencing results obtained thus far are not adequate to draw any conclusions. Further studies are needed to determine the molecular basis for the varying patterns of resistance observed.

CHARACTERISTICS OF DIFFERENTIAL HERBICIDE RESPONSE IN SULFONYLUREA-RESISTANT *KOCHIA SCOPARIA* ACCESSIONS

by

Kailayapillai Sivakumaran

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APPROVAL

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Kailayapillai Sivakumaran

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency and is ready for submission to the College of Graduate Studies.

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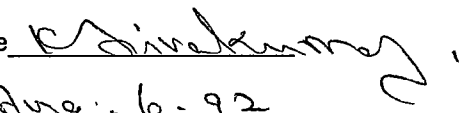
Signature 

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ABSTRACT

Three chlorsulfuron-resistant kochia accessions were tested for levels of resistance to sulfonylurea and imidazolinone herbicides, based on whole plant response and sensitivity of the target enzyme. The Minot and Chester accessions were completely resistant to 175 g ha^{-1} chlorsulfuron, and I_{50} values for the Chester accession ranged from 22-fold (metsulfuron) to 196-fold (chlorsulfuron) higher than the susceptible Bozeman accession. However, the Chester accession was 1.5- to 2-fold more resistant to five other sulfonylurea herbicides than Minot, as determined by ALS I_{50} values. Levels of cross-resistance to four imidazolinone herbicides did not differ appreciably between the Minot and Chester accessions. The intermediate Power accession displayed only 2- to 5-fold increase in resistance over susceptible Bozeman accession to all sulfonylurea herbicides tested, and was not cross-resistant to imidazolinone herbicides.

The highly variable degrees of ALS resistance and cross-resistance among kochia populations may be due to different mutations in the genes encoding ALS. Putative clones have been isolated to determine the molecular basis for the variable responses. DNA sequencing results obtained thus far are not adequate to draw any conclusions. Further studies are needed to determine the molecular basis for the varying patterns of resistance observed.

CHAPTER 1

LITERATURE REVIEW

The Sulfonylurea Herbicides

The discovery of the herbicidal properties of sulfonylureas in the 1970's represents a major advancement in agricultural chemistry. The sulfonylurea herbicides are characterized by favorable environmental characteristics such as low use rates, low non-target toxicity, excellent crop safety, and flexibility of application timing (Levitt *et al.* 1981). These chemicals belong to a large class of compounds of which over 28,000 have been synthesized and characterized (Hartnett *et al.* 1991), while more than 375 have been patented as herbicides (Brown 1990).

Sulfonylurea herbicides are degraded in soil by chemical hydrolysis and microbial activity (Brown 1990). The adsorption, movement, and degradation of sulfonylurea herbicides are affected by soil pH, organic matter, and clay content (Fredrickson and Shea 1984; Walker *et al.* 1989).

The sulfonylurea herbicides show activity against a wide spectrum of weeds including annuals such as kochia (*Kochia scoparia*) and Russian thistle (*Salsola iberica*), and perennials such as Canada thistle (*Cirsium arvense*) (Donald 1987) and McCartney rose (*Rosa bracteata*) (Meyer and Bovey 1990). Crops in which these compounds are used are tolerant due to their ability to metabolically degrade the herbicide (Sweetser *et al.* 1982).

Mode of Action

Initial studies on the mode of action of sulfonylurea herbicides were conducted using chlorsulfuron. These studies demonstrated that the herbicides were extremely potent and selective inhibitors of plant and bacterial growth (LaRossa and Schloss 1984). Ray (1982a; 1982b) demonstrated that plant cell division was rapidly inhibited after treatment with

chlorsulfuron. Growth inhibition of *Salmonella typhimurium* on minimal media but not on complete media led LaRossa and Schloss (1984) to examine several biosynthetic pathways, including those for branched chain amino acids and their respective enzymes. Likewise, studies conducted by Shaner and Reidey (1986) revealed that supplementation of herbicide-treated maize cell cultures with the three amino acids leucine, isoleucine and valine alleviated the herbicidal symptoms. These and other physiological studies conducted with *S. typhimurium* suggested that the target site of sulfonylurea herbicides is the enzyme acetolactate synthase (ALS), also referred to as acetohydroxyacid synthase (AHAS) (EC 4.1.3.18), which catalyzes the first step in branched chain amino acid biosynthesis (Chaleff and Mauvais 1984). ALS is required for the biosynthesis of two acetohydroxy acids: it catalyzes the condensation of pyruvate either with a second molecule of pyruvate to yield acetolactate or with 2-oxobutyrate to yield acetohydroxybutyrate (cited in Stidham and Singh 1991). Carbon flow to branched chain amino acids is regulated via feedback inhibition of ALS (Miflin 1971). Like a majority of plant amino acid biosynthetic enzymes, ALS is nuclear-encoded and localized in the chloroplast (Muhitch *et al.* 1987).

ALS requires the presence of three co-factors which appear to be essential for correct enzyme functioning. Thiamine pyrophosphate (TPP) and divalent magnesium activate the enzyme, while flavin adenine dinucleotide (FAD) acts as a stabilizing factor with no apparent role in catalysis (Muhitch *et al.* 1987; Schloss *et al.* 1984; Schloss *et al.* 1988). Absorption spectra of FAD bound to ALS change during catalysis: these changes were diminished by sulfometuron methyl, but substitution of reduced FAD ($FADH_2$) for FAD caused an increase in the binding constant for sulfometuron methyl (Schloss 1984). Therefore, sulfometuron methyl binding must occur proximal to the site of FAD binding and near the binding site of the second pyruvate (LaRossa and Schloss 1984).

In addition to the sulfonylureas, four other structurally unrelated classes of herbicides also target ALS. These include the imidazolinones (Shaner *et al.* 1984), triazolopyrimidines

(Schloss *et al.* 1988), sulfonylcarboxamides (Crews *et al.* 1989), and N-phthalyl-L-valine anilides (Huppatz and Casida 1985). The triazolopyrimidines appear to interact with plant ALS in a manner similar to the sulfonylurea and imidazolinone herbicides (Schloss *et al.* 1988). Imidazolinone and sulfonylurea herbicides have high levels of activity at very low use rates due to their high specificity and activity towards the plant enzyme (Hartnett *et al.* 1991). Both herbicide classes are slow, tight binding, uncompetitive inhibitors of plant ALS (Muhitch *et al.* 1987). Little is known about interactions of the other two classes of herbicides with ALS (Shaner 1991).

Sulfonylurea and imidazolinone herbicides may compete for the same binding site on the ALS enzyme (Schloss *et al.* 1988). Alternatively, Saxena and King (1988) reported that sulfonylurea-resistant *Datura innoxia* mutants were hypersensitive to imazaquin, indicating that the binding sites may not overlap. These observations suggest the possibility of separable functional sites of action for sulfonylurea and imidazolinone herbicides on ALS (Saxena and King 1990). Recent studies indicate that the binding site of imidazolinone herbicides on the enzyme is distinctly different from that of sulfonylurea herbicides (Sathasivan *et al.* 1991). Their studies showed that a single point mutation at nucleotide 1958 of the ALS encoding sequence in *Arabidopsis thaliana* (*imr 1*) resulted in imidazolinone resistance, but not cross-resistance to sulfonylurea herbicides. Hence, it has been suggested that the point mutation may not cause a significant change in secondary or tertiary structure of ALS, but may instead cause a minor alteration in electrical charge or a steric hindrance at the herbicide-binding site on the ALS molecule resulting in resistance to the herbicide (Sathasivan *et al.* 1991). Likewise, Hattori *et al.* (1992) suggested that the cross-resistance observed in *A. thaliana* could be due to multiple mutations (*imr 1, csr 1*) each conferring resistance to one class of herbicide. However, this question cannot be fully resolved until the crystal structure of ALS is known. Binding domains of the two herbicide classes may still overlap due to secondary and tertiary structure of the enzyme formed by protein folding (Sathasivan *et al.* 1991).

There are up to six ALS isoenzymes in microorganisms which differ in their sensitivity to sulfonylurea and imidazolinone herbicides, as well as feedback inhibition patterns (Schloss *et al.* 1988). In plants, Chaleff and Bascomb (1987) demonstrated the presence of two genes encoding ALS in the tetraploid species *Nicotiana tabacum*: the genes are 99.3% homologous at the amino acid level (Lee *et al.* 1988). Hence, it was suggested that the two isoenzymes may be very similar and expressed at similar levels in the plant (Chaleff and Bascomb 1987). ALS in the diploid species *A. thaliana* (Haughn and Somerville 1986) and *Brassica campestris* (Swanson *et al.* 1988) is encoded by a single gene. The allotetraploid *Brassica napus* contains two or more genes for ALS (Rutledge *et al.* 1991).

The ALS gene sequence from prokaryotic and eukaryotic organisms is conserved to a great degree. Comparisons of deduced yeast and bacterial ALS amino acid sequences revealed three regions of high homology (Falco *et al.* 1985). In higher plants, sequence analysis of *A. thaliana* and *N. tabacum* ALS genes showed that they were highly conserved, with 75% and 85% homology at the DNA and amino acid levels, respectively (Mazur *et al.* 1987b). The 5' end of the coding sequence is not conserved between these two species, this portion of the gene encodes a chloroplast transit sequence (Jones *et al.* 1985). In fact, the majority of the divergence observed among plant ALS genes occurs in the transit peptide region (Hartnett *et al.* 1991). The transit sequences contain only 27% identical amino acid residues, with about 50% homology at the DNA sequence level (Mazur *et al.* 1987a). Hydropathy analysis of the deduced transit peptide sequences indicates that they share a number of similar hydrophobic and hydrophilic domains, thus implying that amino acid polarity in these regions may be more important than the protein primary structures (Mazur *et al.* 1987a).

Mechanism of Plant Resistance to ALS-Inhibiting Herbicides

Herbicide resistance in weeds or crop plants may be categorized into three general mechanisms: 1) modification of the site of action, 2) altered metabolism and 3) limited uptake

or translocation. Mechanisms 1 and 2 account for all known examples of resistance in plants to ALS inhibitors (Shaner 1991). However, the predominant mechanism observed is an altered ALS which is no longer sensitive to the inhibitors (Shaner 1991; Chaleff and Mauvais 1984; Wiersma *et al.* 1989; Sathasivan *et al.* 1991). *N. tabacum* transformed with an altered ALS gene from *A. thaliana* encodes for about 20% of total ALS activity. This fraction was highly resistant to chlorsulfuron (Haughn *et al.* 1988). However, in the absence of herbicide, the amount of ALS activity was observed to be consistently lower than in wild type tissue; the reason for reduced levels of ALS activity in transgenic lines is not known (Haughn *et al.* 1988). There have been no reported cases of increased herbicide resistance among plants or cells selected on ALS inhibitors due to altered uptake or translocation, or increased levels of metabolism (Shaner 1991).

DNA sequence analysis of a sulfonylurea-resistant *A. thaliana* mutant showed that the resistant ALS gene contained a single base substitution, resulting in a proline to serine substitution at position 197 (Haughn *et al.* 1988). A resistant tobacco mutant was shown to contain the above mutation plus a second mutation leading to a leucine to tryptophan substitution at position 573 (Lee *et al.* 1988). This double mutant was cross-resistant to both sulfonylurea and imidazolinone herbicides. In *Escherichia coli,* a resistant ALS gene contained a single base mutation at position 574 causing an alanine to valine substitution (Yadev *et al.* 1986).

Proof of the mode of action of sulfonylurea herbicides came from a combination of genetic and biochemical studies. Studies conducted with herbicide-resistant mutants of *S. typhimurium* (LaRossa and Schloss 1984), *Saccharomyces cerevisiae* (Falco and Dumas 1985), *N. tabacum* (Chaleff and Mauvais), and *A. thaliana* (Haughn and Somerville 1986) demonstrated that ALS activity was insensitive to herbicide inhibition. Studies conducted with transgenic *N. tabacum* plants carrying herbicide-resistant *A. thaliana* ALS genes have confirmed that ALS is the site of action of these herbicides (Mazur *et al.* 1987b). Resistance to ALS

inhibitors is inherited as a single, partially dominant trait in maize (Newhouse *et al.* 1989), tobacco (Chaleff and Bascomb 1987), *A. thaliana* (Haughn and Somerville 1986), prickly lettuce (*Lactuca serriola*) (Mallory-Smith *et al.* 1989), and *Chlamydomonas reinhardtii* (Winder and Spalding 1988). Homozygous resistant plants are more resistant to the herbicides than heterozygous plants (Shaner 1991).

Kochia

Kochia (*Kochia scoparia*) is an introduced herbaceous annual plant that invades cropland, disturbed sites and waste areas throughout the central U.S. (Eberlein and Fore 1984). Kochia is one of 45 species known in the family Chenopodiaceae (Standley 1916). Due to its ability to compete in a wide range of environments and its efficient mating and seed dispersal systems, kochia is considered a major weed pest in many crop and non-crop situations. Kochia flowers about 8 to 12 weeks after emergence and produces around 14,000 seeds per plant (Thill *et al.* 1991). Seed longevity in soil ranges from 2 to 3 years (Zorner *et al.* 1984). The plant is partially self-fertile and has been reported as predominantly open pollinated (Thill *et al.* 1991). However, field studies conducted by Mulugeta (1991) suggest about 4% outcrossing. Kochia is a diploid plant ($2n = 18$) (Uhrikova 1974). Mature plants can abscise at ground level and tumble for long distances, effectively dispersing seed (cited in Thill *et al.* 1991).

Chlorsulfuron, one of the first commercialized sulfonylurea herbicides, has been widely and continuously used in many areas since its introduction in 1980. Due to long soil persistence and high levels of efficacy, weed resistance to chlorsulfuron has developed in a number of locations in the Northern Great Plains. The first reported case of sulfonylurea-resistant weeds was that of prickly lettuce in 1987 (Mallory-Smith *et al.* 1990), followed by kochia (Primiani *et al.* 1990), common chickweed (*Stellaria media*) (Hall and Devine 1989), and Russian thistle (*Salsola iberica*) (cited in Thill *et al.* 1991). Of these species, kochia accounts for the vast majority of reported resistant populations sites (Thill *et al.* 1991).

Chlorsulfuron-resistant kochia is widely distributed in small grain growing areas, but the degrees of resistance and cross-resistance to other ALS-inhibiting herbicides vary widely among field populations. DeCastro and Youmans (cited in Shaner 1991) reported that the cross-resistance of four chlorsulfuron-resistant kochia populations to four sulfonylurea and three imidazolinone herbicides was highly variable. One population was resistant to all sulfonylureas tested and to imazethapyr and imazequin, while all populations were sensitive to imazapyr. Similarly, sulfonylurea-resistant prickly lettuce and kochia populations were cross-resistant to imidazolinone herbicides (Thill *et al.* 1991). However, the degree of cross-resistance varied with the source of the weed populations.

Reduced vigor and ecological fitness may be an intrinsic feature of herbicide resistance, and may be considered as the "cost" or "penalty" for resistance (Gressel and Segel 1982). However, the generalization of this phenomenon has been questioned by Rubin *et al.* (1985) and Chauvel and Gasquez (1991). Chauvel and Gasquez (1991) showed that photosynthetic potential and growth parameters of triazine-resistant populations were at least comparable to those found in susceptible populations, both under competitive and non-competitive conditions. Likewise, very little difference in fitness was found among three isogenic biotypes of triazine-resistant *Chenopodium album* (Gasquez 1991).

Studies conducted by Thompson *et al.* (1992) and Christoffoleti and Westra (1992) showed only slight differences between sulfonylurea-resistant and susceptible kochia accessions with respect to growth rate, biomass production, leaf area and seed production. In greenhouse studies the susceptible accession produced an average of about 13,000 seeds versus 11,000 seeds for the resistant accession (Thompson *et al.* 1992). However, shoot height, shoot and stem diameter, and leaf and stem dry weight were slightly greater in the resistant accessions (Thompson *et al.* 1992). Overall, the results suggest that sulfonylurea-resistant kochia accessions are not less fit than susceptible accessions (Christoffoleti and Westra 1992).

Herbicide Resistant Crops

The significant progress made in introducing herbicide resistance traits into crops by conventional breeding methods and genetic engineering has been the focus of attention in both academic institutions and industry in recent years (Rubin 1991). These achievements have been greatly aided by the progress made in understanding the genetic, biochemical and physiological bases of the herbicides' modes of action and selectivity mechanisms, and the understanding and expertise developed in regenerating plants from cultured cells and tissues (Rubin 1991). The high economic value of herbicides coupled with the cost and complications of new compound registration have encouraged academic and private institutions to pursue research in this area (Mazur and Falco 1989).

Several methods of incorporating herbicide resistance traits into agronomic crops have been employed with varying degrees of success. The methods used to date are: 1) selection at the whole plant, cell, or organelle level, 2) hybridization of crops with resistant weedy relatives, and 3) gene transfer through molecular techniques.

Selection

Genetic analysis of the differential response of crops, grasses and weed species to several herbicides has shown that genes for herbicide tolerance may have evolved before the domestication of cultivated crops (Snape *et al.* 1991). Identification of tolerant germplasm coupled with conventional plant breeding methods have been successful in selecting for increased herbicide resistance levels in perennial turf and pasture grasses (Johnston and Faulkner 1991). *In vitro* selection methods at the cell, tissue, protoplast, and microspore level have also been exploited to select for herbicide resistance. The potential of these methods was demonstrated by the recent selection of maize mutants resistant to cyclohexanedione, aryloxyphenoxy propionate and imidazolinone herbicides: resistance was stably expressed in regenerated plants and their progeny (Newhouse *et al.* 1990; Gronwald *et al.* 1989). *In vitro* selected imidazolinone-resistant maize varieties were released in spring of 1992 (cited in Dyer

et al. 1992).

Hybridization

Early efforts to transfer naturally-occurring herbicide resistance from weed biotypes to related crop species involved the transfer of atrazine resistance. Traditional sexual crosses were employed to transfer maternally-inherited atrazine resistance from *Brassica campestris* to Brassica crops such as canola, oilseed rape, and broccoli (Souza Machado and Hume 1987). In spite of the reduction in yield and quality due to reduced photosynthetic efficiency, triazine-resistant canola cultivars are grown in Canada in locations where weeds are difficult to control by other means (cited in Rubin 1991). A similar approach was used to transfer triazine resistance from green foxtail (*Setaria viridis*) to the related crop foxtail millet (*Setaria italica*) (Darmency and Pernes 1985).

Gene transfer through molecular techniques

A greater understanding of mechanisms of action of herbicides in microorganisms and higher plants at the molecular level has led to significant progress in identifying and transferring genes for resistance into valuable crops. Three techniques have been used so far with success (Rubin 1991).

(a) Transfer of genes encoding insensitive target enzymes. Mutant ALS genes encoding insensitive ALS enzymes were identified and introduced into tobacco, tomato, sugar beet, oilseed rape and other crops (Mazur and Falco 1989; Hartnett *et al.* 1991). The resulting transgenic plants showed varying degrees of resistance to ALS inhibiting herbicides, depending on the type of mutation (Rubin 1991). Several transgenic crop species were shown to be resistant in greenhouse and field trials (Mazur and Falco 1989). Similarly, glyphosate-resistant plants were generated by transferring mutant *S. typhimurium aroA* genes which encode a less sensitive 5-enolpyruvyl-shikimate 3 phosphate synthase (EPSPS) enzyme (Padgette *et al.* 1989).

(b) Overexpression of genes encoding target enzymes. Overexpression of the enzyme targeted by a herbicide results in an increase in the number of target sites available for herbicide inhibition: the net result is that proper pathway functioning may still occur in the presence of the herbicide. Transgenic glyphosate-resistant plants were produced by transferring and overexpressing wild type *aroA* genes from bacteria and plant sources. The EPSPS enzyme was over-produced by about 20-fold due to increased gene expression from the cauliflower mosaic virus (CaMV) 35S promotor (Padgette *et al.* 1989). Introduction of an alfalfa mutant glutamine synthetase gene with a CaMV 35S promotor into tobacco conferred a limited level of tolerance to bialaphos (glufosinate) (Botterman *et al.* 1991). A limiting aspect of this approch is that high levels of stable resistance are usually not obtained.

(c) Introduction of genes encoding detoxifying enzymes. This is a strategy to improve tolerance to a herbicide by introducing genes from other organisms encoding enzymes that break down the herbicide (Rubin 1991). The *bar* gene, which confers resistance to bialaphos, was isolated from *Streptomyces hygroscopicus* and transferred to several crops (Botterman 1989). Similarly, the *bxn* gene, which encodes for a nitrilase responsible for bromoxynil hydrolysis, was isolated from the soil bacterium *Klebsiella pneumonia* subspecies *ozanea* and transferred to tobacco: the resulting transgenic plants demonstrated high levels of resistance (Stalker *et al.* 1988). The *tfd*A gene which encodes a monoxygenase enzyme involved in the breakdown of 2,4-D was isolated from the soil bacterium *Alcaligenes eutrophus* and introduced into tobacco, resulting in transgenic lines with moderate levels of resistance to 2,4-D (Streber and Willmitzer 1989).

Creation of herbicide-resistant crops through biotechnology has been the focus of intense debate. A primary concern is the possibility of transgene escape from crops through hybridization with related wild species (Darmency *et al.* 1991). Since in some cases resistance is inherited as a dominant or partially dominant trait, gene flow by pollen could spread the trait (Rubin 1991). However, all crops are naturally tolerant to some herbicides, and no cases have

been documented of gene flow of tolerance from crops to associated weeds (Dyer *et al.* 1992).

A second concern about herbicide-resistant crops is the possible escape of a resistant crop from cultivation to become a weed, as was the case for non-engineered proso millet (*Panicum milliaceum*) (Bough and Cavers 1989). However, weediness is generally thought to result from many genes and hence it is unlikely that introduction of one or a few foreign genes could cause a crop to become a weed (Dyer *et al.* 1992). Other concerns relating to this issue involve the possibility of altered or novel secondary products produced by transgenic plants, and the increased selection pressure for herbicide resistant weeds that will accompany herbicide resistant crop use (Dyer *et al.* 1992).

Objectives of Study

Although the mechanism of chlorsulfuron resistance in one kochia accession was shown to be due to an insensitive ALS (Saari *et al.* 1990), the physiological basis for differential response to ALS-inhibiting herbicides among other chlorsulfuron-resistant kochia populations is unknown. Hence, the first objective of this study was to conduct a comprehensive analysis of the sensitivity of four kochia accessions to sulfonylurea and imidazolinone herbicides at the enzyme level. The second objective was to determine the molecular basis of resistance in kochia by isolating and sequencing the susceptible and resistant alleles.

CHAPTER 2

WHOLE PLANT AND TARGET ENZYME SENSITIVITY

Introduction

Studies conducted by DeCastro and Youmans (Shaner 1991) showed that four accessions of chlorsulfuron-resistant kochia varied in their sensitivity to sulfonylurea and imidazolinone herbicides. While the mechanism of chlorsulfuron resistance in one kochia accession was shown to be due to an insensitive ALS (Saari *et al.* 1990), the basis for differential response to ALS-inhibiting herbicides among kochia populations is unknown. Hence, the objectives of this study were to (1) determine the whole plant and target enzyme response of four kochia accessions to sulfonylurea and imidazolinone herbicides, (2) determine the inhibitory dose which reduces enzyme activity by 50% (I_{50}) for each accession, and (3) compare enzyme sensitivity levels with whole plant responses.

Materials and Methods

Plant material

In the fall of 1990, kochia seeds were collected and bulked from 20 plants in fields near Chester, Power, and Bozeman in Montana and from Minot, North Dakota. The fields near Chester and Minot had been treated with chlorsulfuron for at least 3 consecutive years prior to seed collection, while the Bozeman accession was from an untreated site. Seeds were stored at 4°C until use. For greenhouse studies, seeds were planted 0.5 cm deep in 40 x 80 cm trays containing potting mixture (Ficon Sunshine Mixture 1):soil:sand (1:1:1). Plants were grown at 25 ± 2°C under natural light supplemented with metal halide lamps (240 μE $m^{-2}sec^{-1}$) to provide a daylength of 15 hours. Plants were fertilized (Peters 20:20:20) weekly.

Whole plant sensitivity

To determine whole plant sensitivity to ALS-inhibiting herbicides, 3-week old (3 to 5 cm tall, 4 to 5 leaf stage) kochia plants were treated with herbicides at 1X and 10X the normal field use rates. Herbicides used (1X field rates in parentheses) were chlorsulfuron (17.5 g ha^{-1}), triasulfuron (33.5 g ha^{-1}), metsulfuron methyl (112 g ha^{-1}), and thibenuron (140 g ha^{-1}). Herbicides were applied with a flat fan 8002E nozzle in a spray volume of 87 L ha^{-1} at 276 kPa using a moving nozzle sprayer. Three weeks after herbicide application, plants were visually rated for injury, harvested at the soil surface, oven dried at 70° C for 48 hours, and weighed. The experiment was conducted twice in a randomized complete block design with four replications. Data were subjected to analysis of variance, and pooled over both experimental repetitions since there was no between-experiment interactions. Means of the four accessions within herbicide rate combinations were analyzed separately using the LSD test ($p \leq 0.05$). Data are reported as percent reduction in biomass compared to the untreated controls of each accession.

Target enzyme sensitivity

ALS extraction and assay was carried out as described in Saari *et al.* (1990) with the following modifications. Leaves were collected from 5 week-old plants (10 to 15 cm tall with 20 to 30 leaves) and immediately frozen in liquid nitrogen. Leaves (10 g fresh weight) were homogenized using a Brinkmann Polytron in 50 mL extraction buffer (Appendix I). The mixture was sparged with nitrogen gas for 15 minutes, filtered through six layers of cheesecloth, and centrifuged at 27,000 X g for 20 minutes at 4° C. The supernatant was saturated with 25% $(NH_4)_2SO_4$, centrifuged, and the supernatant saturated up to 50% with $(NH_4)_2SO_4$ to precipitate the ALS enzyme. Protein extract containing ALS activity was collected by centrifugation at 20,000 X g for 15 minutes at 4° C. The pellet obtained was dissolved in 2 mL of extraction buffer without polyvinylpolypyrollidone, antifoam-A, and anion exchange resin. The resuspended enzyme extract was dialyzed against 2 L of the same buffer for 2 hours at 4° C.

The desalted enzyme extract was used immediately for assay.

ALS activity was assayed as described (Ray 1984) with the following modifications. The final assay mixture contained 50 μL enzyme extract and varying concentrations of the herbicides in the assay buffer (Appendix I) in a reaction volume of 0.5 mL. After incubation at 30°C for 1 hour, the reaction was terminated by the addition of 25 μL 12 N H_2SO_4 and centrifuged in a microfuge for 3 minutes. The supernatant was transferred to new tubes and incubated at 60°C for 15 minutes. Following by the addition of 50 μL of 0.5% (w/v) N-(aminoiminomethyl)-N-methylglycine (creatine) (in water) and 0.5 mL of 50% (w/v) α-naphthol (freshly prepared in 2.5 N NaOH), the reaction was incubated at 60 °C for 15 minutes. Absorbance was measured at 525 nM (Varian tectrometer, Series 634) to determine the amount of acetolactate formed. Total protein content of samples was determined using the Bio-Rad protein assay (Bio-Rad catalog # 500 0006). Specific activity of ALS was calculated using an extinction coefficient of 6.5 mM^{-1} cm^{-1} for acetoin (LaRossa and Schloss 1984). Herbicides were prepared daily from technical grade chemicals as 0.1 mM stock solutions in 1 mM NaOH (chlorsulfuron, triasulfuron, metsulfuron methyl, chlorimuron, sulfometuron methyl, and thibenuron), or in distilled water (imazapyr, imazamethabenz, imazethapyr, and imazequin).

ALS activity data are presented as percent of untreated control activities for each accession. Regression equations of these data were used to calculate I_{50} values as described by Ray (1984), but using the MSUSTAT MRegress program (Version 4.12, developed by Richard E. Lund, Montana State University). I_{50} is defined as the herbicide concentration required to inhibit ALS enzyme activity by 50%. Approximate standard errors associated with I_{50} values were calculated as described (Carlson *et al.* 1983) using

$$SE(I_{50}) = SE\ (y/I_{50})[b_1 + 2(b_2)I_{50}]^{-1}$$

where y = herbicide concentration, b_1 = linear coefficient, and b_2 = quadratic coefficient. Data are reported as averages of two experiments conducted with different plant material, with two

determinations from enzyme extracts for each experiment. Specific activities of ALS from Chester, Minot, Power, and Bozeman accessions were 1.56, 2.75, 1.78, and 1.63 nmoles acetolactate mg protein^{-1} min^{-1}, respectively.

Results and Discussion

Table 1 shows the response of four kochia accessions to treatment with four sulfonylurea herbicides. The resistant Chester and Minot accessions were completely resistant to chlorsulfuron, even at 175 g ha^{-1}. Dry weight of the Chester accession was reduced by about 25% by all herbicides and rates tested, except thibenuron at 140 g ha^{-1}.

Table 1. Dry weights (percent of control) of Bozeman, Power, Chester, and Minot kochia accessions 3 weeks after treatment with four sulfonylurea herbicides at 1X and 10X field application rates.

Accession	Dry Weight (Percent of Control[1])							
	Chlorsulfuron		Triasulfuron		Metsulfuron		Thibenuron	
	1X	10X	1X	10X	1x	10x	1X	10X
Bozeman	51	13	18	8	42	11	21	15
Power	55	33	33	19	37	19	31	25
Chester	101	100	74	75	72	74	96	70
Minot	100	98	44	40	45	43	46	38
$LSD_{0.05}$	4.7	5.0	6.7	9.3	4.9	4.3	4.5	6.8

[1] Percent of dry weight of untreated controls for each accession.

In contrast, the resistant Minot accession was more sensitive to other sulfonylurea herbicides, since dry weight was reduced by more than 50% by the same treatments. The

Power accession generally showed an intermediate response to all treatments, except at the 1X dose of chlorsulfuron, where dry weight was not significantly different from the susceptible Bozeman accession. Thus, the Power accession displayed a low level of resistance to all sulfonylureas tested, while the Minot and Chester accessions were differentially cross-resistant to these closely related herbicides.

At the 1X herbicide doses, dry weights of the susceptible Bozeman accession ranged from 18% (triasulfuron) to 51% (chlorsulfuron) of untreated plant dry weights. The relatively low level of growth reduction by these treatments is most likely a reflection of the time after treatment that plants were harvested. Even though no growth occurred after treatment, plants were still green and turgid after 3 weeks. Treated susceptible plants may survive longer than 1 month under greenhouse conditions.

To compare whole plant response of kochia accessions with *in vitro* ALS sensitivity, ALS activity was assayed in the presence of the four sulfonylurea herbicides tested in Table 1 plus sulfometuron methyl and chlorimuron (Figure 1). ALS from the resistant Chester and Minot accessions was highly resistant to chlorsulfuron, and was not completely inhibited even at the 10 μM concentration (Figure 1a). As seen from the whole plant experiments, ALS from the Power accession displayed an intermediate level of sensitivity to chlorsulfuron, while ALS from the Bozeman accession was very sensitive to this herbicide. ALS sensitivity to the other sulfonylurea herbicides generally followed the same pattern (Figures 1b-1d), although ALS from the intermediate Power accession was only slightly less sensitive to sulfometuron and chlorimuron inhibition than the susceptible Bozeman accession (Figures 1e and 1f).

Regression equations of data in Figure 1 were used to calculate I_{50} values for the six herbicides (Table 2). In all cases, the Chester accession displayed the highest resistance levels of the four accessions. I_{50} values for the Chester accession ranged from 22-fold (metsulfuron) to 196-fold (chlorsulfuron) higher than the susceptible Bozeman accession. In addition, ALS from the Chester accession was generally 1.5- to 2-fold more resistant than enzyme from the

Minot accession. I_{50} values for the Power accession were again intermediate, averaging about 3-fold greater than the Bozeman accession, while remaining substantially below I_{50} values for the resistant Minot and Chester accessions.

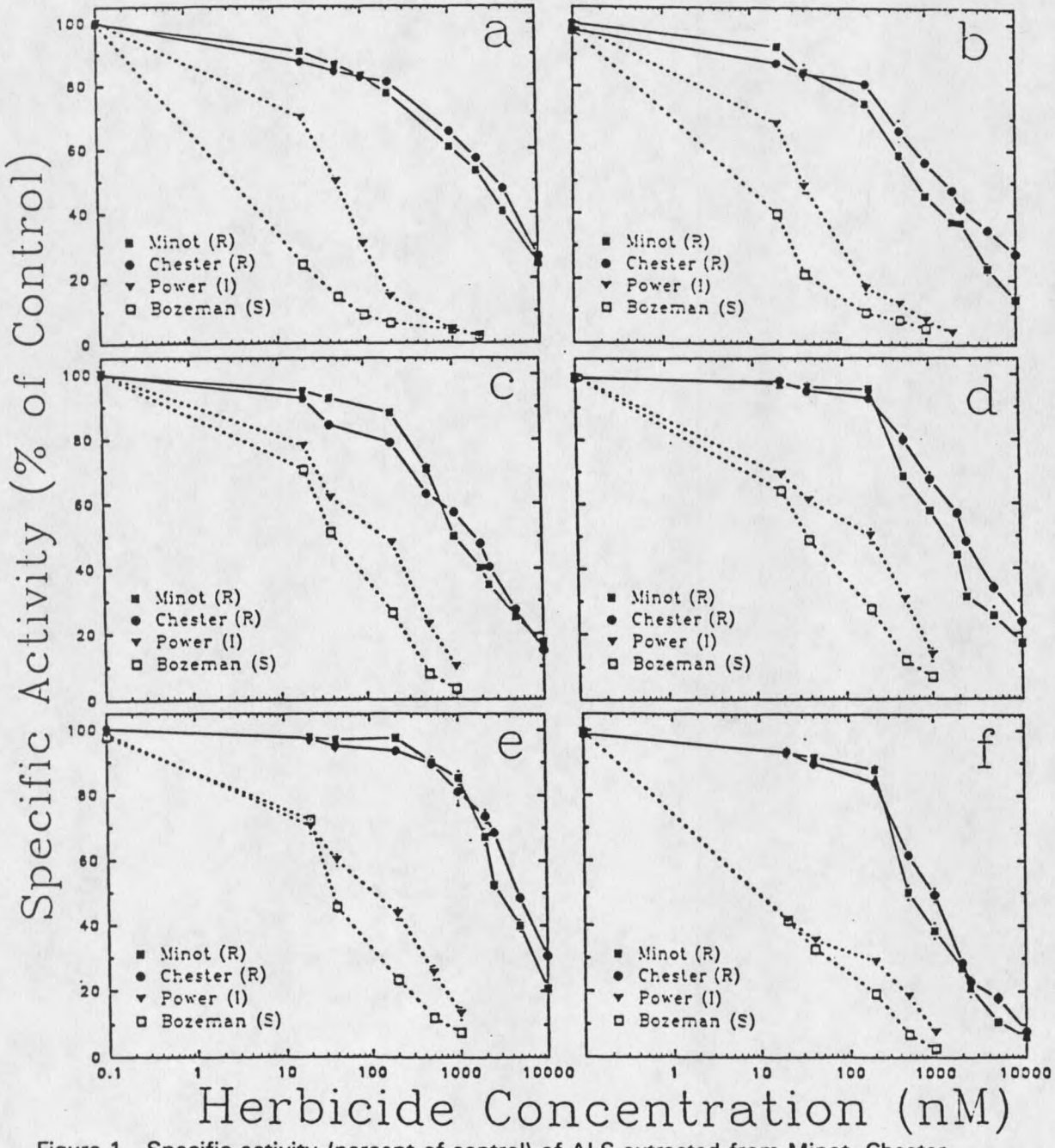

Figure 1. Specific activity (percent of control) of ALS extracted from Minot, Chester, Power, and Bozeman kochia accessions assayed in the presence of (a) chlorsulfuron, (b) triasulfuron, (c) metsulfuron, (d) thibenuron, (e) sulfometuron, or (f) chlorimuron. Vertical bars are SE of means.

Table 2. I_{50} values for six sulfonylurea herbicides of ALS extracted from Bozeman, Power, Chester, and Minot kochia accessions.

	I_{50} Values (nM)					
Accession	Chlorsulfuron	Metsulfuron	Chlorimuron	Thibenuron	Sulfometuron	Triasulfuron
Bozeman	17 (0.5)[1]	89 (7.6)	48 (4.2)	82 (5.1)	84 (6.0)	21 (1.5)
Power	53 (2.0)	213 (8.7)	90 (11)	227 (41)	200 (12)	114 (12)
Chester	3342 (347)	2010 (142)	1475 (217)	2878 (226)	5195 (544)	2244 (172)
Minot	2475 (165)	1872 (88)	1027 (125)	1562 (94)	3583 (350)	1665 (129)

[1] SE of I_{50} values

To determine cross-resistance of kochia accessions to imidazolinone herbicides, ALS sensitivity to four members of this family was determined (Figure 2). ALS from the resistant Chester and Minot accessions was generally less sensitive to herbicide inhibition than enzyme from the other accessions. However, levels of resistance to the imidazolinones were much lower than those observed for the sulfonylurea herbicides. Response of ALS from the Power accession was very similar to ALS sensitivity of the susceptible Bozeman accession, indicating little if any increase in resistance to imidazolinone herbicides. Whole plant response to imidazolinones generally followed the patterns seen at the enzyme level (data not shown).

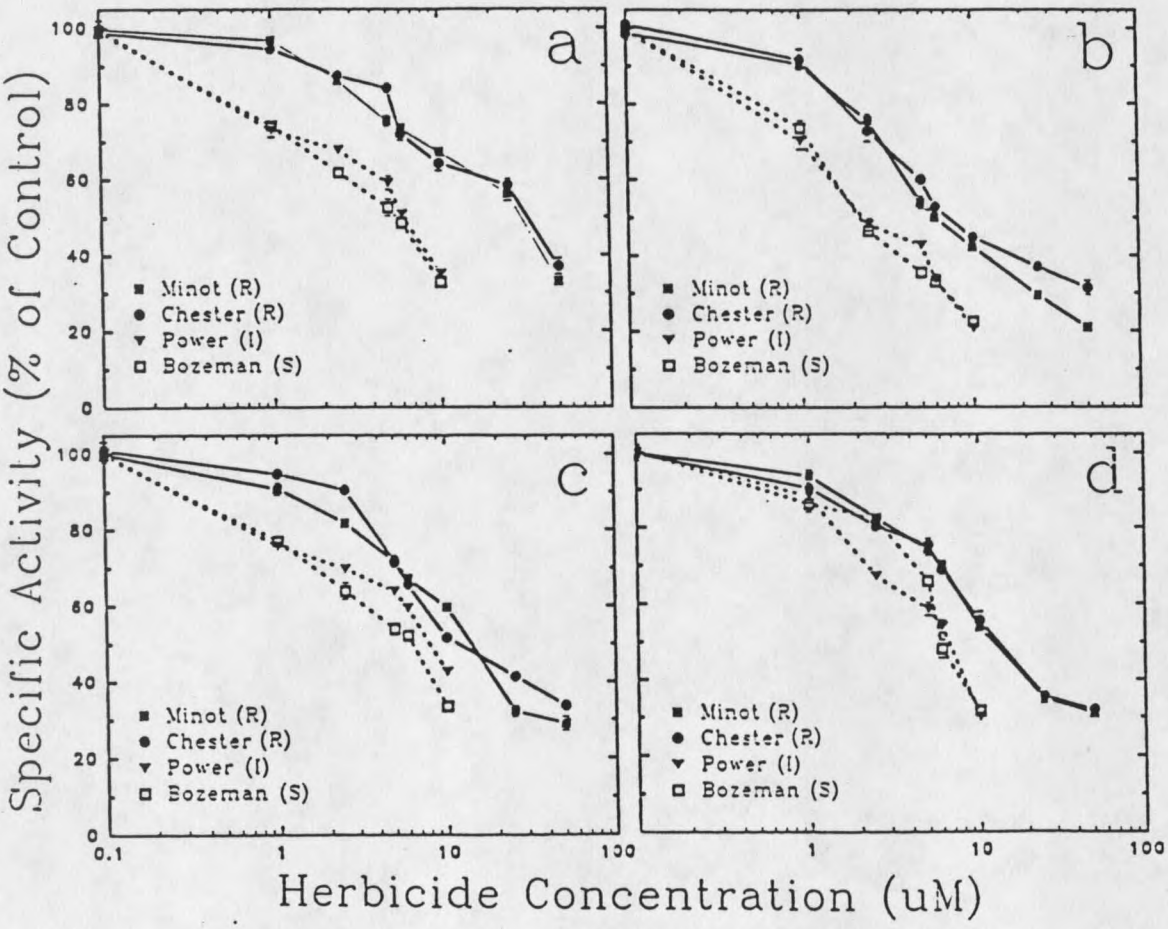

Figure 2. Specific activity (percent of control) of ALS extracted from Minot, Chester, Power, and Bozeman kochia accessions assayed in the presence of (a) imazapyr, (b) imazamethabenz, (c) imazethapyr, or (d) imazaquin. Vertical bars are SE of means.

Regression equations of data in Figure 2 were used to calculate I_{50} values (Table 3). I_{50} values for imidazolinone herbicides of ALS from the resistant Chester and Minot accessions were significantly higher than the susceptible Bozeman accession, although levels of resistance were only about 1.5-fold (imazethapyr) to 6-fold (imazapyr) higher than Bozeman. In contrast to sensitivity differences observed between the Chester and Minot accessions for sulfonylurea herbicides, these lines displayed very similar levels of sensitivity to the imidazolinones. I_{50} values for the intermediate Power accession were generally similar to the Bozeman accession, indicating no substantial increase in resistance to imidazolinone herbicides.

Table 3. I_{50} values for four imidazolinone herbicides of ALS extracted from Bozeman, Power, Chester, and Minot kochia accessions.

	I_{50} Values (μM)			
Accession	Imazapyr	Imazamethabenz	Imazethapyr	Imazequin
Bozeman	6.0 (0.57)[1]	2.4 (0.03)	7.4 (0.17)	5.8 (0.02)
Power	5.9 (0.09)	1.7 (0.05)	5.6 (1.00)	7.0 (0.36)
Chester	37.2 (1.00)	7.3 (0.15)	12.0 (0.67)	12.7 (0.71)
Minot	36.5 (0.93)	5.8 (0.10)	15.6 (0.83)	12.3 (0.72)

[1] SE of I_{50} values.

These results demonstrate differential sensitivity of kochia accessions to sulfonylurea and imidazolinone herbicides at the whole plant and target enzyme levels. Similar results have been reported for four kochia populations (three from ND and one from TX) tested for cross-resistance at the whole plant level (Shaner 1991). All populations were resistant to the selective agent chlorsulfuron, and to thifensulfuron methyl. As observed in the current results, resistance to sulfometuron methyl and metsulfuron was quite variable among the populations. However, in contrast to the current results, only one population was cross-resistant to both imazethapyr and imazaquin while all populations were sensitive to imazapyr (Shaner 1991). In other studies, sulfonylurea-resistant kochia and prickly lettuce displayed different levels of

cross-resistance to imidazolinone herbicides, depending on the source of the resistant weed population (Thill *et al.* 1991).

In general, selection for sulfonylurea and/or imidazolinone resistance in the field or laboratory has resulted in highly variable patterns of cross-resistance. Maize lines selected for imidazolinone resistance showed varying degrees of cross-resistance to sulfonylureas as well as differences in sensitivity among individual imidazolinones (Newhouse *et al.* 1990). Similarly, several differing patterns of cross-resistance were observed among mutants of *Chlamydomonas reinhardtii* (Winder and Spalding 1988) and *Datura innoxia* (Saxena and King 1990) after *in vitro* selection. These results together with the present data on resistant kochia accessions lead to the following conclusions: 1) levels of cross-resistance among field-selected weed species do not follow any predictable patterns, and 2) large scale resistant weed management strategies should not be extrapolated based on the characteristics of only one or a few resistant weed types.

The basis for differential levels of cross-resistance among various plant species probably lies in the nature of mutation in gene(s) encoding ALS. In yeast, mutations in at least ten sites in *ILV2* confer sulfonylurea resistance, and mutations in three corresponding sites in plant genes likewise lead to resistance (Mazur and Falco 1989). Substitutions at Pro 196 in the *N. tabacum* SuRA allele (Lee *et al.* 1988) or the analogous Pro 197 site in the *A. thaliana csr1* allele (Haughn and Somerville 1986) confer selective resistance to sulfonlylurea herbicides. A second allele of *csr1* (designated *imr1* and *csr1-2*) has recently been identified in the *A. thaliana* GH90 mutant (Haughn and Somerville 1990) that confers resistance to imazapyr, but not to sulfonylurea herbicides (Sathasivan *et al.* 1991). The basis for imidazolinone resistance was identified as a substitution from Ser to Asn at residue 653 of ALS. Combining these two herbicide-specific mutations in one chimeric ALS gene conferred cross-resistance to both herbicides in transgenic tobacco (Hattori *et al.* 1992). Thus, it appears that mutations in widely separated regions of the ALS coding sequence may give rise to either herbicide-specific

resistance or varying patterns of cross-resistance. Given the high degree of genetic variability associated with many weed species (Warwick 1991), it seems clear that several combinations of single and multiple mutations may be selected for under field conditions.

The resistant Chester and Minot kochia accessions show a high degree of resistance to chlorsulfuron and the other sulfonylurea herbicides tested. However, the Chester accession is generally more resistant than the Minot accession to these herbicides, a difference that may be due to the type of mutations in their respective ALS genes. In another sulfonylurea-resistant kochia accession, sequence analysis of a 234 bp region within the ALS gene demonstrated the presence of a point mutation leading to an amino acid substitution at Pro 113 (Guttieri *et al.* 1992). Although I do not know which specific mutations occur in the resistant Chester and Minot accessions, apparently they confer significantly different levels of resistance to the sulfonylureas, without altering levels of cross-resistance to the imidazolinones. The intermediate response of the Power accession is also of interest, in that the response may be due to a third type of mutation conferring only low levels of sulfonylurea resistance and essentially no imidazolinone cross-resistance. Resistance level differences among these accessions was not due to altered amounts of the ALS enzyme, since ALS specific activity was similar in all cases. It is unlikely that altered uptake, translocation, or metabolism contributed to the ALS resistance differences measured here, since these processes were not affected in other sulfonylurea-resistant kochia accessions (Saari *et al.* 1990; Thill *et al.* 1991).

If herbicide resistance initially arises from scattered individual plants, then the gene flow (pollen and seed dispersal) characteristics of each species will influence the makeup of resistant populations. Since resistance to ALS inhibitors appears to be inherited as a simple, partially dominant trait (Newhouse *et al.* 1990), weed species may develop homogeneous resistant populations (outcrossing, high seed dispersal) or highly variable lines within resistant populations (selfing, low seed dispersal). In the case of kochia, due to open pollination and high seed dispersal (Thill *et al.* 1991) homogeneous resistant populations are likely to occur.

A better understanding of this variability and the underlying genetic mechanisms is clearly needed in order to develop more effective strategies to manage resistant weeds and prevent further outbreaks.

CHAPTER 3

ISOLATION AND SEQUENCING OF PUTATIVE GENOMIC CLONES

Introduction

Sulfonylurea, imidazolinone, and triazolopyrimidine herbicides may compete for a common binding site on the enzyme, based on herbicide-enzyme binding studies using the bacterial ALS (Schloss *et al.* 1988). However, this may not be the case in plants, since several spontaneous and chemically induced mutant plant enzymes displayed varying levels of cross-resistance to ALS-inhibiting herbicides (Hall and Devine 1990; Saxena and King 1988). Differences in cross-resistance may indicate the presence of multiple mutations in different regions of the gene encoding ALS. Decastro and Youmans reported that the cross-resistance patterns of four chlorsulfuron-resistant kochia populations to sulfonylurea and imidazolinone herbicides were highly variable (Shaner 1991). These studies suggest that the herbicides may be interacting with non-overlapping binding sites on the enzyme (Sathasivan *et al.* 1991), and that there may be separable structural sites of action on the enzyme for the two herbicide classes (Saxena and King 1990). Since levels of resistance and cross-resistance varied in the Power, Chester, and Minot kochia accessions (Chapter 2), I wanted to determine the molecular basis for the observed differences.

Characterizing of the mutation(s) in genes encoding ALS which may have occurred in resistant kochia accessions will help enhance our understanding of the interaction between the sulfonylurea and imidazolinone herbicides and the ALS enzyme. Second, a better understanding of the causes and effects of resistance at the molecular level may help us to develop better weed control programs. Understanding the molecular basis of resistance in weed species may also help in developing better herbicide-resistant crops.

The primary objective of this study was to identify the specific mutation(s) in ALS that led to different levels of resistance and cross-resistance observed in the Chester, Minot, and