Investigations into the mechanism of triallate and difenzoquat resistance in wild oats
by Anthony John Kern

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Agronomy
Montana State University
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Abstract:
Wild oats (Avena fatua L.) are a serious weed competitor in small grain growing areas of North America and the world. One of the most effective herbicides used for wild oat control in these crops is triallate, and its continuous use in the Fairfield region of Montana has selected for populations of wild oats which are no longer controlled by the herbicide. Many of these triallate-resistant (R) wild oats are also resistant to the chemically unrelated herbicide difenzoquat. The objectives of these studies were to characterize resistance levels and investigate potential mechanisms of resistance in an inbred line.

An inbred R wild oat line selected through two generations of triallate treatment was shown in greenhouse and Petri dish dose response experiments to be 17-fold and 64-fold more resistant to triallate and difenzoquat, respectively, than susceptible (S) lines. The R line was also cross-resistant to diallate.

Uptake and translocation patterns of 14C triallate were not substantially different between R and S lines.

Reverse-phase high performance liquid chromatography (HPLC) was used to compare 14C-triallate metabolism patterns in R and S wild oat lines. Triallate was metabolized to one major product (2,3,3-trichloropropene sulfinic acid) and two minor products (unidentified) in R and S wild oats. However, the rate of triallate metabolism was more than 10-fold slower in R than in S lines Because triallate is thought to be activated in vivo through formation of the triallate sulfoxide, dose response tests were conducted to compare the toxicity of triallate sulfoxide in R and S lines. Triallate sulfoxide was equally phytotoxic to both lines, indicating that the mechanism of triallate resistance may be conferred by a decreased rate of sulfoxidation (activation) in R plants.

Difenzoquat uptake, translocation, and metabolism patterns were compared between R and S wild oats using 14C-IabeIed difenzoquat. Slightly increased rates of 14C-difenzoquat uptake and translocation were observed in the R line, which were considered unlikely to confer resistance. However, HPLC analysis indicated that difenzoquat quickly became unextractable in R plants, suggesting the presence of a novel difenzoquat metabolizing pathway and immobilization of the herbicide, possibly in cell wall material.

Because triallate was shown to inhibit lipid biosynthesis in susceptible plant species, the effects of triallate and triallate sulfoxide on lipid and wax biosynthesis in R and S wild oats were compared. Five days after application, wax deposition was dramatically inhibited in S wild oats but not in R. In addition, triallate reduced the amounts of long-chain free fatty acids in S but not R wild oats. However, treatment with triallate sulfoxide inhibited long-chain lipid biosynthesis equally in R and S wild oats. The results further support the idea that decreased synthesis of triallate sulfoxide confers resistance to the herbicide in R wild oats.

INVESTIGATIONS INTO THE MECHANISM OF TRIALLATE AND DIFENZOQUAT RESISTANCE IN WILD OATS

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Anthony John Kern

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The 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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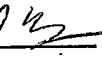
Signature 

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TABLE OF CONTENTS

APPROVAL....ii

STATEMENT OF PERMISSION....iii

ACKNOWLEDGEMENTS....iv

TABLE OF CONTENTS....v

LIST OF TABLES....vii

LIST OF FIGURES....viii

ABSTRACT....x

Chapter

1. INTRODUCTION....1

Non-target Site Resistance....3
Reduced Uptake or Translocation....3
Sequestration....4
Altered Metabolism....5
Target Site Resistance....7
Multiple- and Cross-resistance....9
Herbicide Resistance in Wild Oats....11
Triallate Use and Mode of Action....12
Thiocarbamate Metabolism....14
Difenzoquat Use and Mode of Action....19
Difenzoquat Uptake, Translocation, and Metabolism....23

2. INVESTIGATIONS INTO THE MECHANISM OF TRIALLATE RESISTANCE IN WILD OATS....24

Methods and materials....25
Biotype Selection....25
Greenhouse Dose Response....26

TABLE OF CONTENTS-CONTINUED

Petri Dish Dose Response....26
Triallate Sulfoxide Dose Response....27
Dose Response Statistical Analysis....28
Triallate Metabolism....29
Results and Discussion....31
Greenhouse Dose Response....31
Petri Dish Dose Response....32
Triallate Sulfoxide Dose Response....34
Triallate Metabolism....38

3. INVESTIGATIONS INTO THE MECHANISM OF DIFENZOQUAT RESISTANCE IN WILD OATS....47

Methods and materials....48
Biotype Selection....48
Greenhouse Dose Response....48
Dose Response Statistical Analysis....49
Difenzoquat Uptake....49
Difenzoquat Translocation....50
Difenzoquat Metabolism....51
Results and Discussion....52
Difenzoquat Dose Response Experiments....52
Difenzoquat Uptake....53
Difenzoquat Translocation....54
Difenzoquat Metabolism....57

4. EFFECTS OF TRIALLATE AND TRIALLATE SULFOXIDE ON LIPID BIOSYNTHESIS IN WILD OATS....61

Materials and methods....62
Epicuticular Wax Deposition....62
Analysis of Free Fatty Acids....63
Results and Discussion....64
Epicuticular Wax Deposition....64
Analysis of Free Fatty Acids....67

LITERATURE CITED....70

LIST OF TABLES

Table Page

1. Distribution of ^{14}C triallate and its metabolites in R and S wild oats at various times after treatment....45

2. Translocation of ^{14}C difenzoquat in FG93R22 (R) and SH430 (S) wild oat tissues 12, 24, 48, 72, and 96 hours after application....56

3. Total and extractable radioactivity in R and S wild oats at various times after treatment with ^{14}C difenzoquat....58

4. Fatty acid concentrations in R and S wild oat seedlings 48 hours after treatment with 10 or 100 ppm triallate....68

5. Fatty acid concentrations in R and S wild oat seedlings 48 hours after treatment with 100 or 500 ppm triallate sulfoxide....69

LIST OF FIGURES

Figure Page

1. Chemical structures of EPTC (S-alkyl) and orbencarb (S-benzyl) thiocarbamate herbicides....15
2. Cytochrome P450-mediated sulfoxidation of EPTC....16
3. Glutathione conjugation and secondary metabolism of EPTC, as proposed by Leavitt and Penner (1979)....17
4. Diallate sulfoxide rearrangement yielding carbamoylsulfenyl chloride and chloroacrolein, as proposed by Schuphan and Casida (1979) and Schuphan et al. (1979)....18
5. *In vivo* conjugation of diallate sulfoxide with GSH and subsequent cleavage, yielding a mercapturic acid and dichloroallyl sulfonic acid (from Schuphan et al., 1979)....20
6. Cycling of difenzoquat and the difenzoquat free radical and production of hydrogen peroxide mediated by photosynthetic reducing power....22
7. Growth inhibition of FG93E22 and SH430 wild oat lines by triallate application in the greenhouse....32
8. Growth inhibition of FG93R22 and SH430 wild oat lines by triallate application in Petri dishes....33
9. Growth inhibition of FG93R22 and SH430 wild oat lines by diallate application in Petri dishes....35
10. Growth inhibition of FG93R22 and SH430 wild oat lines by root uptake of triallate and triallate sulfoxide in Petri dishes....37
11. Representative HPLC chromatograms of ^{14}C triallate and metabolites eluted using reverse-phase HPLC....39

LIST OF FIGURES-CONTINUED

Figure Page

12. HPLC chromatograms of Peak I from Figure 11 and TCPSA in the WAX chromatography system.....41

13. Proposed pathway of triallate metabolism in R and S wild oats, including enzymes and cofactors.....42

14. Representative HPLC chromatograms from S and R wild oats 24 hours after treatment with ^{14}C triallate.....44

15. Growth inhibition of FG93R22 and SH430 wild oat lines by greenhouse difenzoquat treatment.....54

16. Uptake of ^{14}C-labelled difenzoquat in FG93R22 and SH430 wild oats.....55

17. Deposition of new epicuticular wax in R and S wild oats five days after treatment with 0.1 kg/ha triallate.....66

ABSTRACT

Wild oats (Avena fatua L.) are a serious weed competitor in small grain growing areas of North America and the world. One of the most effective herbicides used for wild oat control in these crops is triallate, and its continuous use in the Fairfield region of Montana has selected for populations of wild oats which are no longer controlled by the herbicide. Many of these triallate-resistant (R) wild oats are also resistant to the chemically unrelated herbicide difenzoquat. The objectives of these studies were to characterize resistance levels and investigate potential mechanisms of resistance in an inbred line.

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Reverse-phase high performance liquid chromatography (HPLC) was used to compare ^{14}C-triallate metabolism patterns in R and S wild oat lines. Triallate was metabolized to one major product (2,3,3-trichloropropene sulfinic acid) and two minor products (unidentified) in R and S wild oats. However, the rate of triallate metabolism was more than 10-fold slower in R than in S lines Because triallate is thought to be activated *in vivo* through formation of the triallate sulfoxide, dose response tests were conducted to compare the toxicity of triallate sulfoxide in R and S lines. Triallate sulfoxide was equally phytotoxic to both lines, indicating that the mechanism of triallate resistance may be conferred by a decreased rate of sulfoxidation (activation) in R plants.

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Because triallate was shown to inhibit lipid biosynthesis in susceptible plant species, the effects of triallate and triallate sulfoxide on lipid and wax biosynthesis in R and S wild oats were compared. Five days after application, wax deposition was dramatically inhibited in S wild oats but not in R. In addition, triallate reduced the amounts of long-chain free fatty acids in S but not R wild oats. However, treatment with triallate sulfoxide inhibited long-chain lipid biosynthesis equally in R and S wild oats. The results further support the idea that decreased synthesis of triallate sulfoxide confers resistance to the herbicide in R wild oats.

CHAPTER 1

INTRODUCTION

Since the introduction of 2,4-D (2,4-dichlorophenoxyacetic acid) in 1952, selective herbicides have been an important tool in modern agriculture. Herbicides are generally very effective in reducing competition from weeds, and have been an integral part of the so-called "Green Revolution." However, a current threat to the productivity of modern agriculture is the recent advent of weed populations that are resistant to previously effective herbicides, due to altered physiological or biochemical processes which enable the plant to escape injury. Under normal circumstances, wild type individuals of a plant species are capable of tolerating a certain amount of herbicide (herbicide tolerance). Maxwell and Mortimer (1994) defined herbicide resistance as "the inherited ability to not be controlled by a herbicide" when that herbicide is applied at rates substantially higher than rates that normally control the wild type individuals.

In 1970, Ryan reported the first case of herbicide resistance to the photosynthesis inhibitor simazine (6-chloro-N', N'-diethyl-1,3,5-triazine-2,4-diamine) in *Senecio vulgaris* (Ryan, 1970), and during the next twenty years several dozen instances of herbicide resistance have been documented,

involving many weedy plant species and nearly every herbicide class used in agriculture. For example, herbicide resistance has been reported for photosystem II inhibiting herbicides in over 60 weed species (LeBaron, 1991), and for the photosystem I inhibiting herbicides in over 12 species (Preston, 1994). Resistance to the acetolactate synthase inhibiting herbicides was detected less than five years after their introduction in 1982 in *Lactuca serriola* (Mallory-Smith et al., 1990), and since then over a dozen species have developed resistance (Saari et al., 1994). Resistance to the acetyl-coenzyme A carboxylase inhibiting herbicides used for grass control has been reported in nine weed species (Devine and Shimabukuro, 1994). Over 20 weedy species have been reported to be resistant to auxinic herbicides (Coupland, 1994), a class of herbicides with diverse chemical structures that mimic the natural plant growth regulator indoleacetic acid. Mitotic disrupting herbicides, such as the dinitroaniline herbicides, have been used for over two decades and nearly a dozen weeds are now reported to be resistant (Smeda and Vaughn, 1994; Morrison et al., 1989).

The great diversity of weeds developing resistance to many herbicide classes demonstrates the high levels of phenotypic variability present in weedy species. In addition, there is substantial variability in the mechanisms of resistance adopted by weeds to escape injury. These resistance mechanisms can be divided into two classes based on the type of physiological alteration present: non-target site resistance and target site resistance.

Non-target Site Resistance

Plants with non-target site resistance posess altered physiological processes that alter the fate of a herbicide before it can influence the normal biochemical processes that ultimately result in plant death. Generally, non-target site resistance confers levels of resistance that are about 10- to 20-fold higher than normal susceptible biotypes (Hall et al., 1994).

Reduced Uptake or Translocation.

Reduced herbicide uptake (absorption) through the cuticle, cell wall and plasmalemma may cause the applied herbicide to remain on the leaf surface, or slow the rate of absorption into the cell. Resistance due to decreased uptake in wheat (*Triticum aestivum)* has been documented for triasulfuron (2-(2-chloroethoxy)-n-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl) amino]carbonyl] benzene sulfonamide) (Meyer and Muller, 1989) and several *Equisetum* species exhibit tolerance to the nonselective herbicide glyphosate (N-(phosphonomethyl) glycine) due to limited uptake (Marshall et al., 1987). However, there are very few documented examples of resistance due entirely to reduced herbicide uptake (Saari et al., 1994). In most cases where limited uptake plays a role in herbicide resistance, it is coupled with reduced translocation (movement) within the plant to the ultimate site of action. One example of reduced translocation conferring resistance is to the non-selective bipyridilium herbicides paraquat (1:1-dimethyl-4,4'-bipyridinium dichloride) and diquat (6,7-dihydroipyridol[1,2-a:2',1'-

c]pyrazidinium dibromide). Reduced uptake and translocation of paraquat (as measured by autoradiography) have been noted in certain biotypes of *Erigeron philadelphicus*, *Erigeron canadensis* (Tanaka et al., 1986), *Hordeum glaucum* (Bishop et al., 1987), *Conyza bonariensis* (Fuerst et al., 1985), and *Lolium perenne* (Faulkner, 1976). In all cases, paraquat and diquat were taken up and translocated more slowly in the resistant (R) than the susceptible (S) biotypes. Although the mechanism of reduced uptake and translocation appears to be sufficient to confer agronomically important levels of resistance, it has not been observed to be a widespread means of field-selected resistance.

Sequestration

Sequestration (or compartmentalization) of herbicides into cellular vacuoles has been documented for diclofop-methyl (methyl, 2-[4-(2,4-dichlorophenoxy) phenoxy] propanoate) in *Avena fatua* (Devine et al., 1993), for paraquat in certain biotypes of *Hordeum vulgare* (Hart and DiTomaso, 1993), in a 2,4-D resistant biotype of soybean (Schmitt and Sandermann, 1982) and for other auxin-analog herbicides (Davis and Linscott, 1986; and Shaner et al., 1992). In addition, sequestration into cell walls has been proposed as a paraquat resistance mechanism for certain types of *Conyza bonariensis* (Fuerst et al., 1985, Vaughn et al., 1989) and *Hordeum glaucum* (Powles and Cornic, 1987). In *H. glaucum*, several polyamines inhibited paraquat uptake in S biotypes, and the polyamines were taken up more slowly in R biotypes than S biotypes (Preston et al., 1992). The authors proposed that a polyamine and/or paraquat transporter

had been altered in R biotypes, conferring reduced paraquat uptake into individual cells. Paraquat thus excluded may slowly accumulate in extracellular matrices and become bound to cell walls via non-enzymatic processes. Although sequestration has not been proposed as a mechanism of field-selected resistance, Hart et al. (1992) showed that more than 75% of applied paraquat became associated with maize root cell walls within 24 hours after application, indicating that sequestration of the bipyridilium herbicides could confer sufficient levels of resistance to allow plants to escape injury under normal field conditions.

Altered Metabolism

Although the metabolic fates of herbicides are quite diverse across plant species, an increase in the rate of any catabolic step of herbicide breakdown can reduce the concentration of its active form *in vivo*, thus effectively decreasing its phytotoxicity. There are several well-characterized examples of enzymatic steps common to herbicide metabolism, which have been shown to confer resistance in R populations.

Cytochrome P450-mediated aryl- and alkyl-hydroxylation, *O*-dealkylation, sulfoxidation, and deesterification reactions have been reported in R biotypes of several species as proposed mechanisms of metabolism-based detoxification (Brown, 1991; Fonne-Pfister et al., 1990; Frear et al., 1991; Omer et al., 1990). Increased cytochrome P450-mediated herbicide metabolism was shown to confer resistance to the acetolactate synthase-inhibiting herbicides such as chlorsulfuron (2-chloro-N-((4-methoxy-6-methyl-1,3,5-triazin-2-yl) aminocarbonyl)

benzenesulfonamide) in various species (Sweetser et al., 1982), and to the photosystem II inhibiting herbicides simazine and atrazine (Ritter, 1986). In addition, acetyl-coenzyme A carboxylase inhibiting herbicides such as diclofop-methyl undergo aryl hydroxylation in R species, yielding nontoxic metabolites (Jacobson and Shimabukuro, 1984; Zimmerlin and Durst, 1990). Two biotypes of *Lolium rigidum* have been shown to be resistant to a number of photosystem II inhibiting herbicides (including the triazines and phenylureas) due to enhanced N-dealkylation (Burnet et al., 1993; Burnet et al., 1993a), which is thought to be mediated by cytochrome P450-type enzymes. It is not known whether these increased oxidative capabilities are due to a mutant cytochrome P450 with broad substrate specificity, or due to increases in the activity of a number of cytochrome P450 isozymes. Inhibitors of cytochrome P450s such as 1-aminobenzotriazole and piperonyl butoxide not only decrease the rate of herbicide detoxification, but can also cause the plant to revert to susceptibility.

Subsequent reactions that lead to herbicide conjugation with glutathione (Dean et al., 1991), sugars (Gonneau et al., 1988), fatty acids, and other cellular components (Gronwald, 1994) can occur, all of which alter the chemical characteristics of a herbicide and can render it ineffective. For example, *Abutilon theophrasti* biotypes that are resistant to atrazine (6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine) have been shown to have a sixfold greater rate of atrazine conjugation to glutathione (GSH) than S biotypes (Gronwald et al., 1989). The enhanced rate of GSH conjugation was not due to an elevated GSH content, but rather to the overexpression of two glutathione-*S*-

transferase (GST) isozymes that exhibit activity with atrazine. The high natural levels of tolerance to atrazine shown by maize may be due to a similar rapid GST activity (Timmerman, 1989) and a high endogenous GSH content.

Herbicide resistance could also theoretically be conferred through a decreased rate of metabolism. For example, diclofop-methyl is considered a proherbicide because it must be demethylated *in vivo* to be activated, and thus a lack of metabolism would prevent formation of the herbicidally active metabolite of the parent molecule. Although this mechanism has been proposed for a number of herbicides, the phenomenon has not yet been documented in field- or laboratory-selected plants.

Target Site Resistance

Herbicide resistance may be caused by an alteration in the normal binding site of a herbicide (usually an enzyme) that results in decreased binding specificity or affinity. Resistance based on target site alterations has been best documented for photosystem II inhibitors such as the triazines and phenylureas. Tischer and Strotmann (1977) reported that many herbicides in these classes have a common binding site on thylakoid membranes known as the D1 protein or the 32-kDa protein (Barber and Andersson, 1992; Trebst, 1987). Herbicides of these families exert their effect by displacing plastoquinone at the Q_B binding site on the D1 protein, thereby blocking electron flow from Q_A to Q_B (Vermass et al., 1983; Vermass et al., 1984). Current models propose that plastoquinone binding

to the Q_B binding niche involves hydrogen bonding between the carbonyl oxygens of plastoquinone with His215 and Ser264 of D1 (Fuerst and Norman, 1991; Tietjen et al., 1991; Trebst, 1992). In all cases of target site resistance that have occurred in the field, a point mutation in the *psb*A gene (which encodes the D1 protein) (Morden and Golden, 1989) resulted in the substitution of a Gly residue for Ser264; this substitution has been identified as the alteration conferring resistance (Fuerst and Norman, 1991; Mets and Thiel, 1989; Trebst, 1991). This amino acid substitution results in an approximate 1000-fold reduction in the binding affinity for atrazine, and confers an approximate 100-fold increase in atrazine resistance in whole plants (Pfister and Arntzen, 1979). Such dramatic increases in herbicide resistance levels caused by target site mutations are common; similar R/S I_{50} (the herbicide concentration at which enzyme activity is inhibited by 50%) ratios have been documented for target site chlorsulfuron resistance in *Stellaria media* (Devine et al., 1991; Saari et al., 1992), *Lolium rigidum* (Saari et al., 1994), *Kochia scoparia* (Friesen, 1992), and sulfometuron-methyl (methyl 2-[[[[4,6-dimethyl-2-pyrimidinyl) amino] -carbonyl] amino]sulfonyl]benzoate) resistance in *Salsola iberica* (Saari et al., 1992). Herbicide resistance levels based on target site mutations are generally greater than resistance based on non-target site alterations.

Multiple- and Cross-resistance

Evolution of resistant populations under extensive herbicide selection pressure is not surprising (Maxwell and Mortimer, 1994), and illustrates that there is great diversity in resistance mechanisms and resistance levels among species and even within small populations. Among the many cases of herbicide resistance documented in recent years, an alarming and serious agricultural threat is the advent of populations that are resistant to more than one herbicide class. These populations are said to have multiple resistance, defined as the "expression (within individuals or populations) of more than one resistance mechanism, endowing the ability to withstand herbicides from different chemical classes" (Hall et al., 1994). Typically, plants exhibiting multiple resistance possess two or more distinct resistance mechanisms, which may include both non-target site and target-site based resistances. The most well-characterized example of multiple resistance is based on a set of *Lolium rigidum* populations from Australia (Hall et al., 1994). One particular population is resistant to 24 different herbicides, encompassing nine different chemical classes (the aryloxyphenocypropanoates and cyclohexanediones, which are acetyl-coenzyme A carboxylase inhibitors; the sulfonylureas and imadazolinones, which inhibit acetolactate synthase; the dinitroaniline herbicides, which appear to inhibit mitosis by preventing spindle microtuble formation (Hess, 1987; Morejohn et al., 1987); the carbamates, which are photosystem II disruptors; the thiocarbamates (see discussion below); and the chloracetamides and isoxalolidinones, which

may inhibit biosynthesis of fatty acids, proteins, isoprenoids, and flavonoids through conjugation with sulfhydryl-containing compounds such as acetyl coenzyme A (Fuerst, 1987).

The mechanisms that confer multiple resistance to such a wide range of herbicides include: 1) the "membrane recovery response" which, although poorly characterized, allows meristematic tissue to quickly recover trans-membrane proton gradients after application of the aryloxyphenocypropanoate and cyclohexanedione herbicides (Hausler et al., 1991); 2) enhanced herbicide metabolism which decreases the toxicity of diclofop-methyl, possibly by increased glutathione conjugation (Christopher et al., 1993); 3) increased chlorsulfuron metabolism, which appears to be cytochrome P450- mediated (Christopher et al., 1991; Cotterman and Saari, 1992); and 4) target site resistances due to mutations in the genes encoding acetyl-coenzyme A carboxylase and acetolactate synthase (Matthews et al., 1990).

A somewhat less complicated mechanism by which weedy species evolve resistance to more than one herbicide is known as cross resistance. In species exhibiting cross resistance, a single mutation confers resistance to more than one herbicide of the same class and mode of action. Populations of *Eleusine indica* which developed resistance to trifluralin (2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl) benzenamine) were also resistant to seven other dinitroaniline herbicides, and exhibited R/S I_{50} ratios of over 1,000 (based on mitotic indices), even though the population had been selected in the field by trifluralin only (Mudge et al., 1984). Populations of *Setaria viridis* in Canada which also

developed resistance to trifluralin in the field were cross-resistant to five other dinitroaniline herbicides, although R/S I_{50} ratios were much lower (Smeda et al., 1992).

Herbicide Resistance in Wild Oats

Wild oats (*Avena fatua* L.) are a major weed problem causing significant yield reductions in small grain crops in the U.S. and Canada (Thurston and Phillipson, 1976). One of the most effective herbicides for controlling wild oats in spring wheat and barley is triallate (*S*-(2,3,3-trichloro-2-propenyl) bis(1-methylethyl)carbamothioate), a selective preemergence thiocarbamate herbicide. In certain cropping systems, the chemically unrelated bipyridilium postemergence herbicide difenzoquat (1,2-dimethyl-3,5-diphenyl-1*H*-pyrazolium) is also used for effective wild oat control. Both herbicides are widely used, and in certain areas triallate has been used annually for over 20 consecutive years since its introduction in 1972. Despite early reports of natural variation and increased levels of tolerance to triallate in wild oat populations repeatedly subjected to sublethal doses (Jana and Naylor, 1982, Thai et al. 1985), the herbicide has been used successfully for over two decades.

Field reports of poor triallate performance beginning in 1991 and 1992 led O'Donovan et al. (1994) to investigate the response of Canadian wild oat collections to triallate and several other herbicides. Seedlings from R collections exhibited about a 10-fold increase in resistance levels to triallate. In addition, the

R collections were about 40-fold more resistant to difenzoquat than S controls. At about the same time, wild oat field collections from near Fairfield, MT were tested due to complaints of non-performance. Malchow et al. (1993) reported that 61% of the fields sampled contained triallate-resistant wild oats. Many of these populations were tested at a later date and shown to be cross-resistant to difenzoquat (Kern et al., 1994). Although some wild oat lines with elevated tolerance to triallate were previously shown to be slightly cross-resistant to the aryloxyphenoxypropanoate herbicide diclofop-methyl and various cyclohexanedione herbicides (Thai et al., 1985), the collections from Canada (O'Donovan et al., 1994) and Montana (Kern et al., 1994) showed normal susceptibility to diclofop.

Triallate Use and Mode of Action

Triallate is used as a preplant incorporated herbicide for control of wild oats in spring wheat and barley. Although triallate is somewhat phytotoxic to most small grains, deep seeding (below the herbicide treatment zone) allows the seeds to germinate and grow through treated soil without injury. When applied at the recommended field rate (1.1 kg/ha), triallate normally provides greater than 80% control of wild oats. Wild oat seedlings germinate normally but usually do not emerge due to severe inhibition of shoot growth, most likely because of elongation of the first internode, which places the shoot meristem in contact with treated soil.

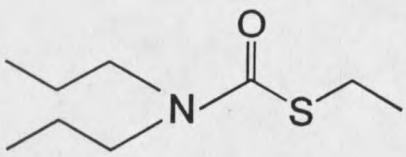
A definitive mode of action has not been established for triallate or the class of herbicides to which it belongs, the thiocarbamates. There is some evidence that other thiocarbamates such as EPTC (*S*-ethyl dipropylcarbamothioate) and diallate (*S*-2,3-dichloro-2-propenyl) bis(1-methylethyl) carbamothioate) inhibit gibberellin biosynthesis (Wilkinson, 1983; Wilkinson 1986), but this effect is more likely due to general cell damage following inital effects of the herbicide and not to specific enzyme inhibition. Other research has shown that thiocarbamates inhibit the biosynthesis of surface lipids such as suberin and waxes (Barrett and Harwood, 1993; Harwood et al., 1989; Harwood and Stumpf, 1971; Kolattukudy and Brown, 1974; Still et al., 1970). As early as 1966, Gentner noted that the total amount of wax deposition on developing cabbage leaves was reduced when treated with EPTC, and several reports confirming this have since been published (Leavitt et al., 1978; Wilkinson and Hardcastle, 1969). Gas chromatography was used to characterize wax biosynthesis inhibition by the thiocarbamates (Wilkinson, 1974; Still et al., 1970). In nearly all cases, species having a large primary alcohol component in their epicuticular waxes with carbon chain lengths greater than 20 were the most severely affected by this treatment. Epicuticular waxes are synthesized by condensation of 16- or 18-carbon fatty acids such as palmitate and stearate with malonyl-CoA by acyl-coenzyme A elongases in an NADPH-dependent reaction. Inhibition of acyl-coenzyme A elongases may thus be a primary site of action of the thiocarbamates (Bolton and Harwood, 1976; Harwood and Stumpf, 1971; Harwood, 1992; Harwood, 1994; Kolattukudy and Brown, 1974). However, total

fatty acid biosynthesis was also strongly affected by triallate treatment in some studies (Harwood et al., 1971; Hawke and Leech, 1987).

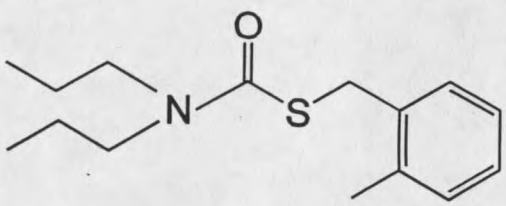
The sulfoxide derivatives of thiocarbamate herbicides have been shown to covalently bind sulfhydryl-containing molecules and consequently have been classified as carbamoylating agents (Lay and Casida, 1976; Leavitt and Penner, 1979). Nonenzymatic *in vitro* carbamoylation of EPTC sulfoxide to coenzyme A has been documented in several systems (Lay and Casida, 1976; Lay et al., 1975; Leavitt and Penner, 1979). Because of the extensive reactions in which coenzyme A is involved (including biosynthesis of lipids isoprenoids, and flavonoids), carbamoylation and subsequent depletion of coenzyme A could account for the wide variation in biochemical perturbations documented in susceptible species. It thus remains unclear whether triallate inhibits specific enzymes, or if the carbamoylating nature of triallate provides a more general enzyme inhibition.

Thiocarbamate Metabolism

Thiocarbamate herbicides have been shown to be metabolized extensively both in plants and mammals, and several metabolites have been identified. The *S*-alkyl (EPTC) and *S*-benzyl (orbencarb) thiocarbamates (Figure 1) are known to be proherbicides in that they require activation to exhibit herbicidal effects. These compounds undergo metabolic sulfoxidation to form the moderately stable sulfoxide derivatives as shown for EPTC in Figure 2.



EPTC

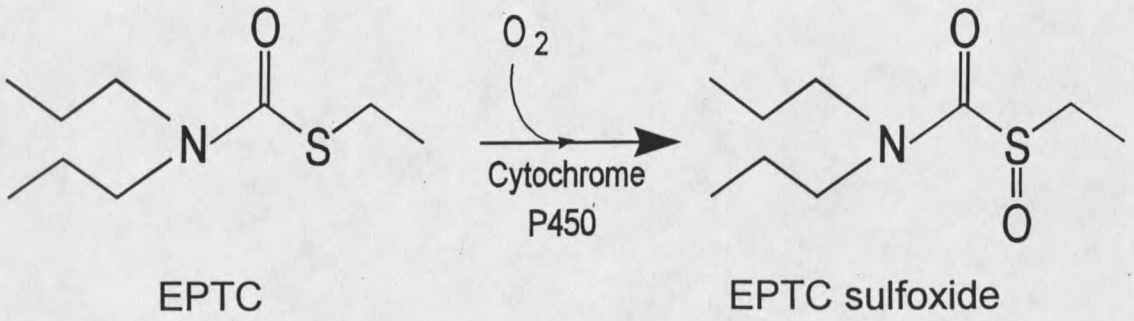


orbencarb

Figure 1. Chemical structures EPTC (*S*-alky) and orbencarb (*S*-benzyl) thiocarbamate herbicides.

After activation, degradation of the *S*-alkyl and *S*-benzyl thiocarbamates has been shown to occur via the sulfoxide intermediates in various systems including mice, rats, and corn (Casida et al., 1975; Hubbell and Casida, 1977; Wilkinson, 1983; Lay and Casida, 1979). Although oxygen is required for EPTC sulfoxidation in maize microsomal fractions (Casida et al., 1975; Jablonkai and Hatzios, 1994), which suggests a cytochrome P450-mediated reaction, subsequent metabolism was not shown to be enzyme dependent. Using ^{3}H-labeled GSH, Leavitt and Penner (1979) showed that EPTC sulfoxide is cleaved during conjugation with GSH, indicating a possible degradation pathway producing the mercapturic acid derivative of EPTC and the sulfinic acid (Figure 3).

The rate of glutathione conjugation with EPTC did not appear to be



EPTC EPTC sulfoxide

Figure 2. Cytochrome P450-mediated sulfoxidation of EPTC.

dependent on GST activity in crude enzyme extracts of maize leaves (Carringer et al., 1978). However, the *S*-alkyl and *S*-benzyl thiocarbamates butylate(S-ethyl bis (2-methylpropyl) carbamothioate), cycloate (S-ethyl cyclohexylethylcarbamothioate), EPTC, molinate (S-ethylhexahydro-1H-azepine-1-carbothioate), pebulate (S-propyl butylethylcarbamothioate), and vernolate (S-propyl dipropylcarbamothioate) were shown to undergo sulfoxidation in a rat microsome system, followed by conjugation with GSH in the liver glutathione transferase system (Casida et al., 1975). After GSH conjugation, subsequent metabolites were not identified. Similarly, Lay and Casida (1976) showed that GSH thiol conjugation with diallate sulfoxide was dependent on GST activity in maize roots. Other *in vivo* work showed that maize seedlings treated with dichloroacetamide herbicide antidotes (which elevate GST activity) rapidly metabolized EPTC sulfoxide in the presence of GSH, indicating that GST activity (dependent on the presence of GSH) increased the rate of sulfoxide cleavage

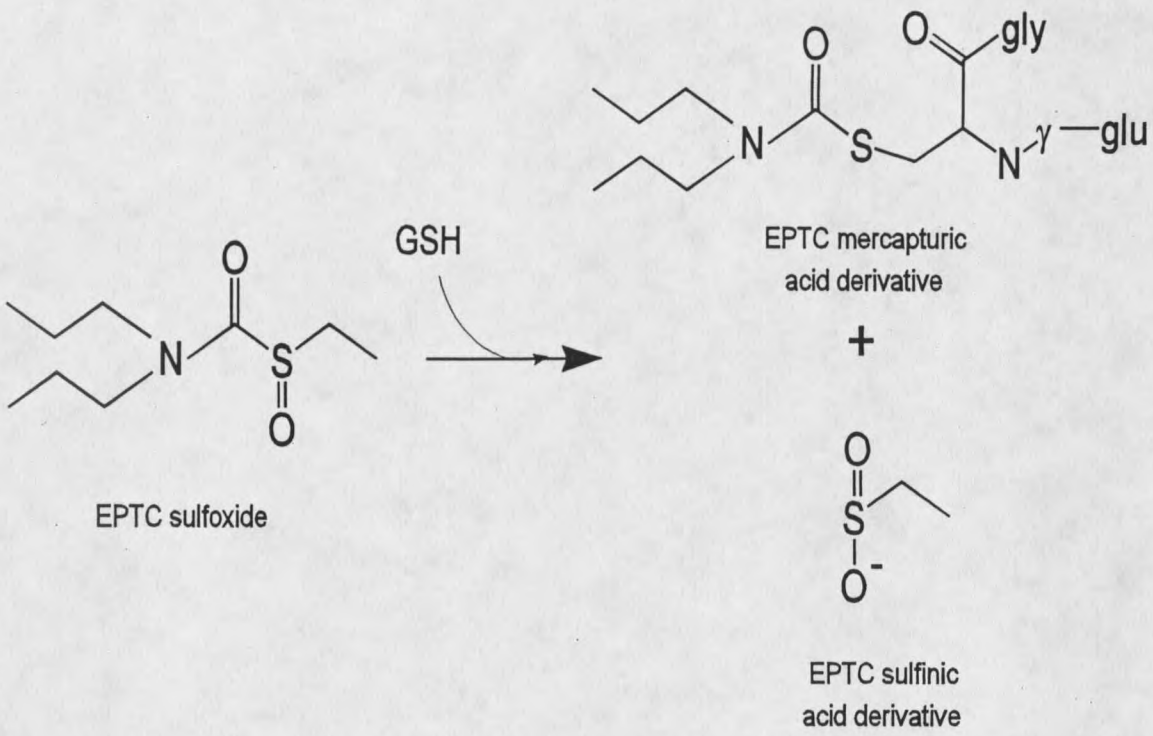


Figure 3. Glutathione conjugation and secondary metabolism of EPTC, as proposed by Leavitt and Penner (1979).

(Carringer et al., 1978).

Metabolism of *S*-chloroallyl thiocarbamates such as diallate and triallate is not clearly understood. While these compounds may undergo sulfoxidation *in vivo* like the *S*-alkyl and *S*-benzyl thiocarbamates, the sulfoxide derivatives have not been isolated from any plant or animal species. Work done by Casida and Lay (1978), Schuphan and Casida (1979a), and Schuphan et al. (1979) showed that the diallate and triallate sulfoxides have half-lives of less than three hours at room temperature. The authors suggested that these primary metabolites could not be isolated in living systems because of immediate *in vivo* conjugation with

various compounds. In addition, the sulfoxide derivatives of *S*-chloroallyl thiocarbamates are unstable immediately after synthesis. Schuphan and Casida (1979a) and Schuphan and Casida (1979b) showed that triallate and diallate sulfoxides undergo a [2,3] sigmatropic rearrangement followed by a 1,2-elimination reaction in organic solvents to yield the carbamoylsulfenyl chloride and chloroacrolein, as shown for diallate in Figure 4.

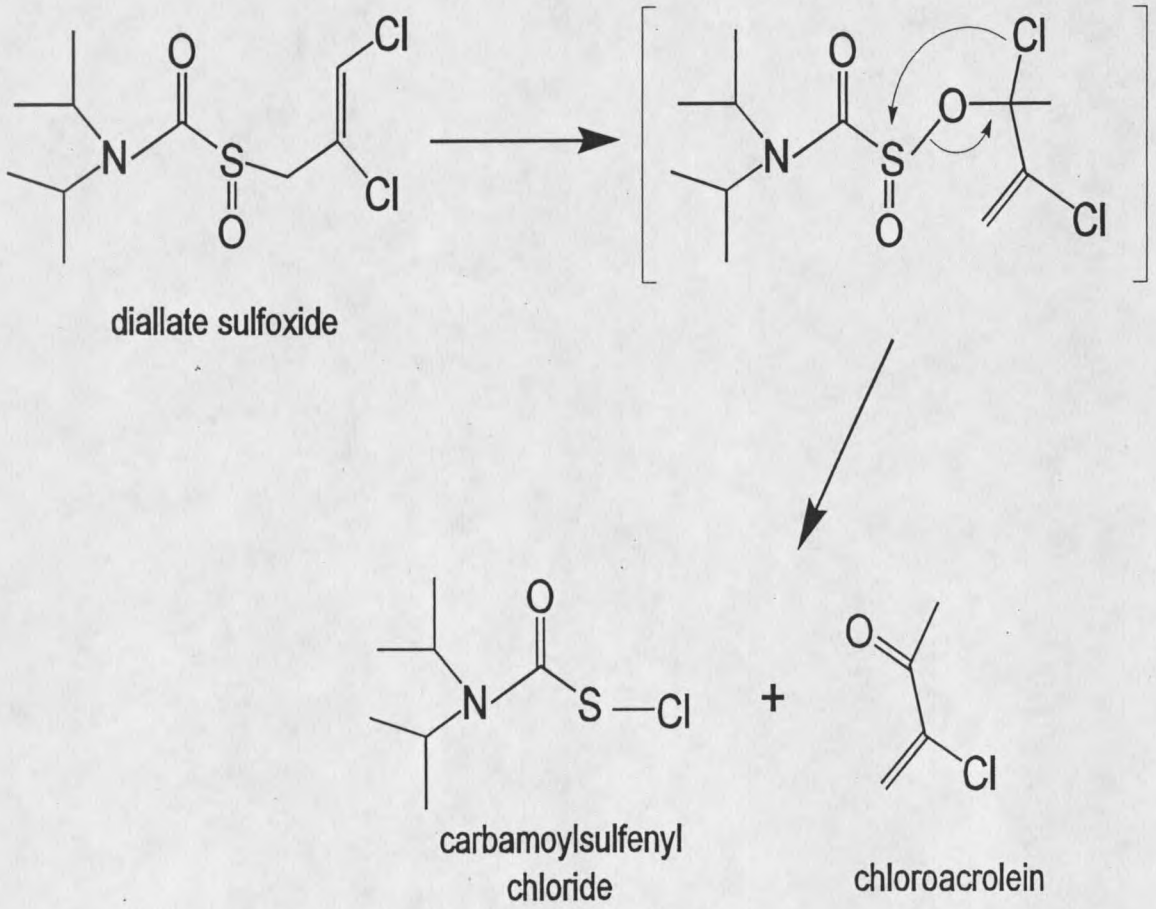


Figure 4. Diallate sulfoxide rearrangement yielding carbamoylsulfenyl chloride and chloroacrolein, as proposed by Schuphan and Casida (1979) and Schuphan et al. (1979).

Diallate sulfoxide was also shown to react with excess GSH in rat liver microsomal systems, forming the relatively stable carbamoyl-GSH derivative, which was subsequently converted to the mercapturic acid derivative of diallate (Schuphan et al., 1979). After GSH conjugation, cleavage of diallate occurred, liberating the unstable sulfenic acid derivative which was quickly converted to the dichloroallylsulfonic acid (Figure 5).

Similar metabolic fates of diallate have been documented in maize (Lay et al., 1975; Hubbell and Casida, 1977). In all biological systems studied, the dichloroallyl sulfonic acid and the mercapturic acid derivative formed after GSH conjugation accounted for more than 80% of the final metabolites (Lay et al., 1975; Hubbell and Casida, 1977).

Difenzoquat Use and Mode of Action

Difenzoquat is used in small grain crops as a late postemergence herbicide for wild oat control. Application is made when wild oats are in the 3- to 5-leaf stage, and 95% control is usually achieved. Sensitive plants treated with difenzoquat generally become stunted, exhibit excessive tillering, and the foliage takes on a deep green color.

Although the chromosome location conferring difenzoquat resistance in hexaploid wheat has been mapped (Leckie and Snape, 1992; Snape et al., 1987), little is known about the mode of action of difenzoquat. Difenzoquat is thought to function *in vivo* like other bipyridilium herbicides, such as paraquat and

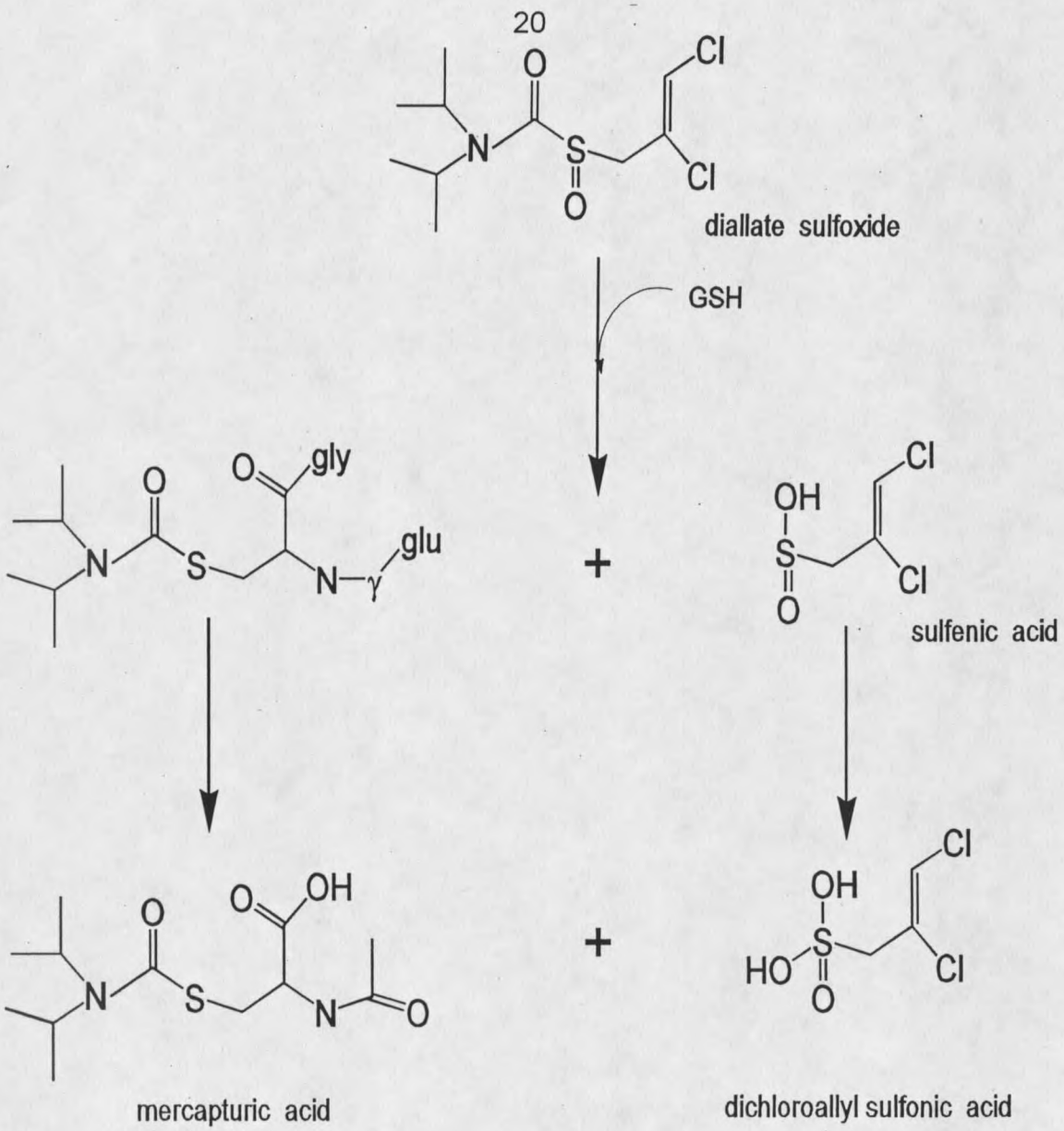


Figure 5. *In vivo* conjugation of diallate sulfoxide with GSH and subsequent cleavage, yielding a mercapturic acid and dichloroallyl sulfonic acid (from Schuphan et al., 1979).

diquat, which exhibit their herbicidal effects by channeling electron flow from photosystem I by continuous cycling through their cationic and anionic forms (Goldbeck and Cornelius, 1986). Electrons are siphoned from an iron center (F_b) away from ferredoxin, and react with difenzoquat to form the bipyridyl cation

radical (Figure 6).

The bipyridyl cation radical is highly unstable and reacts with oxygen to form superoxide, and in the process the bipyridyl cation is regenerated. Subsequent enzymatic reactions involving superoxide (superoxide dismutase) occur and create free hydrogen peroxide. Endogenous levels of Fe^{2+} subsequently catalyze the Fenton reaction (Fridovich, 1983), which ultimately produces free hydroxyl radicals from hydrogen peroxide. These active oxygen species can peroxidize unsaturated molecules in cell membranes and other lipids, and subsequently cause cell death. Levels of hydroxyl radicals increased dramatically after treatment with paraquat (Babbs et al., 1989).

Plants treated with paraquat exhibit rapid contact lesions and cell necrosis, especially in full sunlight, indicative of cellular disruption. Although difenzoquat was shown to cause perturbations in the tonoplast and plasmalemma of cells after application (Thai et al., 1989), difenzoquat exhibits only moderate external contact activity, and produces limited necrotic lesions shortly after application.

Because of additional plant stunting symptoms (Shaner, 1983; Suttle and Hulstrand, 1984), difenzoquat is also thought to have a second herbicidal effect that results in the inhibition of meristematic activity (Shaner, 1983). However, the mechanisms responsible for plant stunting and eventual death remain elusive. Pallett and Caseley (1980) showed that DNA synthesis, as measured by incorporation of ^{14}C-thymidine in the meristematic region of susceptible spring wheat cultivars, was inhibited after difenzoquat treatment. In contrast, the

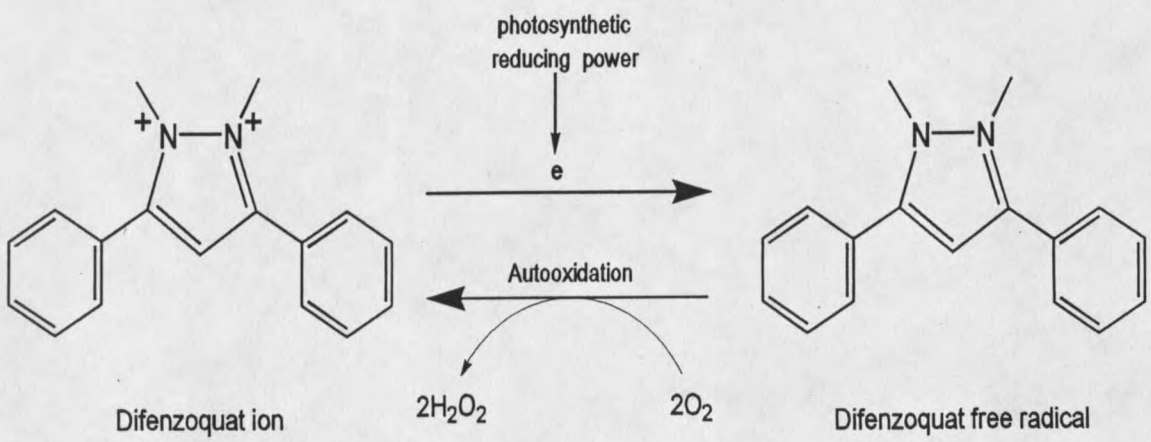


Figure 6. Cycling of difenzoquat and difenzoquat free radical and production of hydrogen peroxide mediated by photosynthetic reducing power. Modified from Ashton and Crafts, 1981.

authors noted no inhibition of DNA synthesis in difenzoquat-tolerant varieties of wheat. The authors proposed that inhibition of DNA synthesis was responsible for reduced cell division in susceptible species, resulting in the characteristic stunting observed after difenzoquat treatment.

Because difenzoquat has been shown to interfere with other electron transfer systems in animals (Kappus, 1986), other studies addressed the effect of difenzoquat treatment on photorespiration. Halling and Behrens (1983) showed that at high concentrations, difenzoquat caused a slight inhibition of photophosphorylation. However, no differences were seen between tolerant and susceptible species in mitochondrial oxidative phosphorylation. The authors proposed that leaf necrosis and cell death may result from a bipyridilium-type electron acceptor activity, if sufficient quantities of difenzoquat are absorbed.

Difenzoquat Uptake, Translocation, and Metabolism

Difenzoquat is absorbed by plants more slowly than other bipyridilium herbicides, although more than 90% of the applied herbicide is taken up after 48 hours (Sharma et al., 1976). Autoradiographic studies using ^{14}C-labelled difenzoquat showed that the majority of applied difenzoquat (greater than 70% by 24 hours after application) remained at the site of treatment, with most of the absorbed radioactivity moving acropetal to the site of treatment (Clipsham, 1985; Sharma et al., 1976). The acropetal movement was most likely an effect of transport in the xylem, and only a limited amount of basipetal translocation due to phloem and/or intracellular transportation occurred. No metabolites of difenzoquat or other bipyridilium herbicides have been reported in any plant species tested (Preston, 1994).

CHAPTER 2

INVESTIGATIONS INTO THE MECHANISM OF TRIALLATE RESISTANCE IN WILD OATS

Wild oat populations resistant to triallate were first documented in Alberta in 1991 (O'Donovan et al.), and shortly thereafter in fields near Fairfield, Montana (Malchow et al. 1993). Because of heavy grower reliance on triallate for wild oat control in spring grains, there are serious concerns about the future efficacy of triallate. These studies were done to determine the mechanism of triallate resistance in wild oats, and may provide valuable information needed to overcome this resistance or to help delay the onset of resistance to herbicides with similar biochemical characteristics.

Work initiated by Colliver et al. (1993) showed that levels of triallate uptake and translocation were slightly lower in R than S plants, but the differences were not judged to be sufficient to explain the degree of whole plant resistance. In addition, the same authors found preliminary evidence that the rate of triallate metabolism was substantially different between R and S plants. Therefore, I began studies designed to more fully characterize the response of R populations to triallate treatment, and to compare the metabolic fate of triallate in

both R and S plants.

Methods and Materials

Biotype Selection

Susceptible (S) wild oat seeds used for experiments were collected from field-grown populations of the nondormant inbred line SH430 (Jana and Naylor, 1976) grown at the Arthur H. Post Research Farm in Bozeman, MT in 1993. Putative S seeds were also collected in 1993 from field borders or fields near Fairfield, MT which had not been treated with triallate for at least 16 months. Putative R seeds used for preliminary experiments were collected from fields near Fairfield, MT in which triallate had been used annually for several years (Malchow et al., 1993) and from plants that had survived treatment with 1.1 kg/ha triallate the preceeding spring. The field collections were tested in preliminary screening experiments to confirm resistance under greenhouse conditions.

Greenhouse soil mix (1:1:1 Bozeman silt loam:washed sand:peat moss, (v/v/v)) was treated with 1.1 kg/ha triallate in 187 L water/ha and mixed thoroughly in an electric soil mixer for 10 minutes. Treated soil was spread uniformly 2 cm deep over 50 seeds that had been placed on top of 5 cm of untreated soil in 55 x 35 x 10 cm flats. Flats were held in the greenhouse with a 14-hr daylength under natural sunlight supplemented with mercury vapor lamps and day/night temperatures of 22/16° C and watered as needed. After 14 days, field collections from which at least 50% of the seedlings emerged and grew

without injury symptoms were considered to be resistant to triallate.

One of these collections, FG93R22, had the highest proportion of R individuals and was used to develop the inbred R line used for all subsequent experiments. Approximately 200 FG93R22 plants surviving the initial greenhouse triallate treatment described above were grown to maturity, selfed, and the progeny (S_1) subjected to an identical triallate treatment. The surviving individuals (about 1000 plants) were again grown, selfed, and their progeny (S_2) used for all subsequent experiments. For all experiments, seeds were peeled (lemma and palea removed by hand) to aid in surface sterilization and enhance germination.

Greenhouse Dose Response

Three rows of 15 caryopses each from lots SH430, FG93R22, two S field collections, and 2 R field collections were placed in flats and treated as described above with 0.0035, 0.07, 0.13, 0.27, 0.55, 1.1, 2.2, or 4.4 kg/ha triallate. After 30 days, seedlings were counted, shoot heights measured, the shoots clipped at the soil surface, and individual shoot fresh weights were measured. There were 3 replicates of 15 seeds each as subsamples in a randomized complete block design and the experiment was repeated once.

Petri Dish Dose Response

Caryopses of inbred lines and field accessions shown to be resistant or susceptible to greenhouse triallate treatment were surface sterilized by shaking

for 15 minutes in 15% (v/v) sodium hypochlorite. After vigorous rinsing for 10 minutes in sterile deionized water, the caryopses were incubated at 22+/-2°C on one sheet of Whatman No. 4 filter paper moistened with 3.5 ml of sterile deionized, distilled water in 25x100 mm Petri dishes in the dark. After 4 days, uniform etiolated seedlings with approximately 1-cm long shoots were selected and treated with formulated triallate (4 EC) or formulated diallate (4 EC) diluted in sterile water. Treatment solutions (1 µl) were placed on the proximal 0.5 mm of the shoot apex with a 10-µl syringe and allowed to dry. For triallate dose response experiments, R and S seedlings were treated with 0, 30, 50, 100, 150, 200, or 250 ppm triallate. For diallate dose response experiments, S seedlings were treated with 0, 1, 5, 10, 25, 35, 50, 75, 100, or 200 ppm and R seedlings were treated with 0, 50, 100, 150, 200, 250, 300, 400, or 500 ppm diallate. Petri dishes were then sealed with Parafilm and incubated at 20°C in the dark. After 7 days, shoot lengths (within or beyond the coleoptile) were recorded. There were 10 replicate seedlings for each treatment, and the experiment was repeated once.

Triallate Sulfoxide Dose Response

Triallate sulfoxide was synthesized from technical grade triallate (Monsanto, 98.9% pure) using the method of Lay and Casida (1979) with the following modifications. A solution of 800 ppm triallate was made in 25 ml distilled deionized water containing 7% octyl phenoxy polyethoxyethanol (Triton X-100, Sigma cat. no. T9284). M-chloroperoxy benzoic acid (Sigma cat no.

C9416) was added in a 1.4 molar excess, the mixture was vortexed for 30 seconds, and the emulsion was incubated for one hour on ice. During the incubation, aliquots were removed after 5, 15, and 60 minutes and used to determine the reaction kinetics by analysis using high performance liquid chromatography (HPLC, see below). Upon completion of the reaction (>95% conversion), the emulsion was diluted in deionized water containing 7% octyl phenoxy polyethoxyethanol. FG93R22 and SH430 seedlings in Petri dishes were treated by adding 0.5 ml triallate sulfoxide solution on the filter paper to give treatment rates of 0, 0.1, 0.5, 1, 5, 10, 50, or 100 ppm triallate sulfoxide. There were two replicate Petri dishes containing six seedlings each as subsamples, and the experiment was repeated once.

Dose Response Statistical Analysis

Data analysis was conducted by Corey Colliver using programs written by Dr. Bruce Maxwell, Department of Plant, Soil, and Environmental Sciences, Montana State University-Bozeman. Prior to logistic analysis, data from each experiment were tested for possible block effects using the general linear models procedure (PROC GLM) of the Statistical Analysis Software. No significant blocking effects were detected ($\alpha = 0.05$) and data were pooled for the log-logistic analysis.

A SAS non-linear models procedure (PROC NLIN) program was constructed for the log-logistic equation (Streibig et al., 1993) as described by Seefeldt et al. (1995). The program compared two biotypes by deriving

appropriate transformations for both sides (Streibig et al., 1993) of the equation and the associated parameter values. Confidence intervals were computed for the parameter values to indicate possible differences between curves (Streibig et al., 1993; Seefeldt et al., 1995), and appropriate GR_{50} values calculated. For all models, the assumptions of parallel curves and equal asymptotes were not rejected.

Triallate Metabolism

FG93R22 and SH430 caryopses were germinated in Petri dishes as described above. Four day-old etiolated seedlings were treated by spotting 1 µl of 1-^{14}C-labelled triallate (1.67×10^3 Bq; sp. act. 3.42×10^6 Bq/mg) on the shoot apex. Plants were harvested after 1, 3, 6, 9, 12, 24, 36, 48, 60, 72, or 96 hours, rinsed for 10 seconds in 60% (v/v) acetone to remove unabsorbed triallate, and five shoots were pooled and homogenized in a 2.0 ml Broeck glass homogenizer in 0.5 ml 60% (v/v) acetone. The crude homogenate was centrifuged at 13,600 x g for 4 minutes at room temperature and the supernatant removed. The supernatant was filtered through 0.22 µm nylon mesh in microspin centrifuge filters at 5000 x g for 5 minutes at 4°C. A 10-µl aliquot from the resulting filtrate was removed and radioactivity present quantified using liquid scintillation counting. Samples were standardized by dilution with 60% acetone to yield 0.5 ml extracts containing 25,000 dpm and subjected to HPLC analysis. There were 3 replications of 5 seedlings each for each time point, and the experiment was repeated once.

Most HPLC analyses used a C18 reverse phase column in 4.6mm x 250mm format[1]. The mobile phase consisted of 95% water:5% acetonitrile on injection which was ramped linearly to 50% water:50% acetonitrile over 10 minutes, and then again ramped to 100% acetonitrile over the next 2 minutes. The gradient programmer[2] and HPLC pump apparatus[3] delivered solvent at 2.0 ml/min and separated radioactive compounds were identified using an in-line scintillation counter[4]. Radioactivity present in individual peaks was quantified using a dedicated integrator[5].

HPLC analysis of highly charged triallate metabolites was conducted using a 4.6 x 125mm Weak Anion Exchange (WAX) column[6] format. The mobile phase consisted of 100% water on injection which was ramped to 90% water:10% 0.75M ammonium phosphate buffer (pH 3.2) over 10 minutes. The HPLC apparatus and gradient programmer[7] delivered solvent at 1.0 ml/min and retained radioactive metabolites were quantified using an in-line scintillation counter[8].

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[1]Lichrosorb RP-18 5U reverse phase HPLC column. Alltech Associates, Deerfield, Il 60015.
[2]Isco model 2369 gradient programmer. Isco, Inc., Lincoln, Ne 68505.
[3]Isco model 2350 HPLC pump. Isco, Inc., Lincoln, Ne 68505.
[4]Beckman model 171 Radioisotope Detector. Beckman Instruments, Inc., Fullerton, CA 92634.
[5]Shimadzu C-R3A Chromatopac dedicated integrator. Shimadzu Corp., Kyoto, Japan.
[6]Partisphere WAX HPLC column. Waters, Inc., Milford, MA 01757.
[7]Waters model 510 HPLC pump and Waters Automated Gradient Controller. Waters, Inc., Milford, MA 01757.
[8]Packard Radiomatic Series A-200 Radiochromatography Detector. Packard, Inc., Downers Grove, IL 60515.

Results and Discussion

Greenhouse Dose Response

The inbred S line SH430 was very sensitive to triallate treatment, with a GR_{50} value (the treatment rate at which seedling growth was reduced by 50%) of approximately 0.13 kg/ha triallate (Figure 7). Because the other S lines and field collections tested exhibited similar visual effects after triallate treatment, SH430 was used as the representative S line for all experiments. S lines were sensitive to doses about 10-fold less than typical field use rates, due to the enhanced efficacy of triallate under greenhouse growth conditions. In contrast, the GR_{50} value for FG93R22 was about 2.2 kg/ha, representing a 17-fold increase in the level of resistance compared to S lines. Typical GR_{50} values for other R lines ranged from 1.3 to 2.2 kg/ha triallate, or a 10- to 17-fold increase in resistance levels (data not shown). These findings show resistance levels in Montana to be slightly higher than those reported for wild oat lines from Canada by O'Donovan et al. (1994), who found a 7- to 12-fold increase in triallate resistance levels in R biotypes from Manitoba.

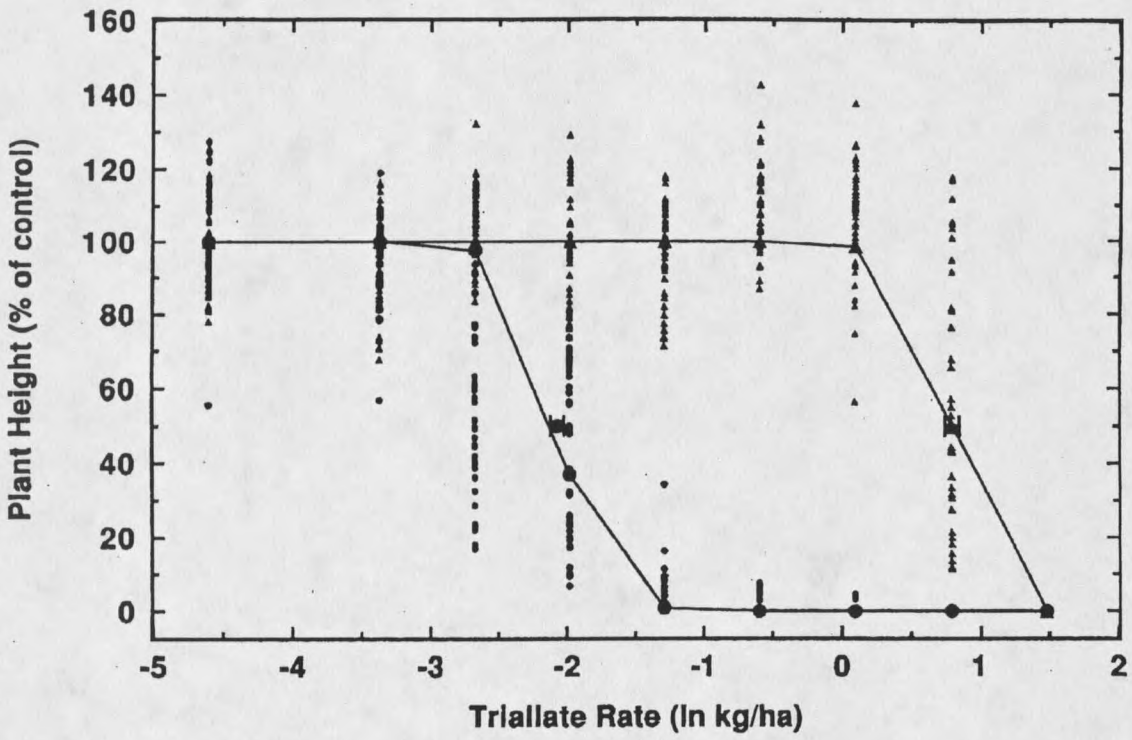

Figure 7. Growth inhibition of FG93R22 (triangles) and SH430 (circles) wild oat lines by triallate application in the greenhouse. Horizontal error bars represent the 95% confidence intervals of the natural log of triallate doses needed to inhibit growth by 50% (±0.006 kg/ha for SH430 and ±0.109 for FG93R22; see Methods and Materials).

Petri Dish Dose Response

Resistance levels demonstrated in the greenhouse were again tested in Petri dish assays in order to confirm the suitability of this treatment method for subsequent experiments. In all lines compared, GR_{50} values and R/S ratios determined in Petri dish dose response assays were two- to three-fold lower than in greenhouse tests. For triallate, R and S GR_{50} values were estimated at 180

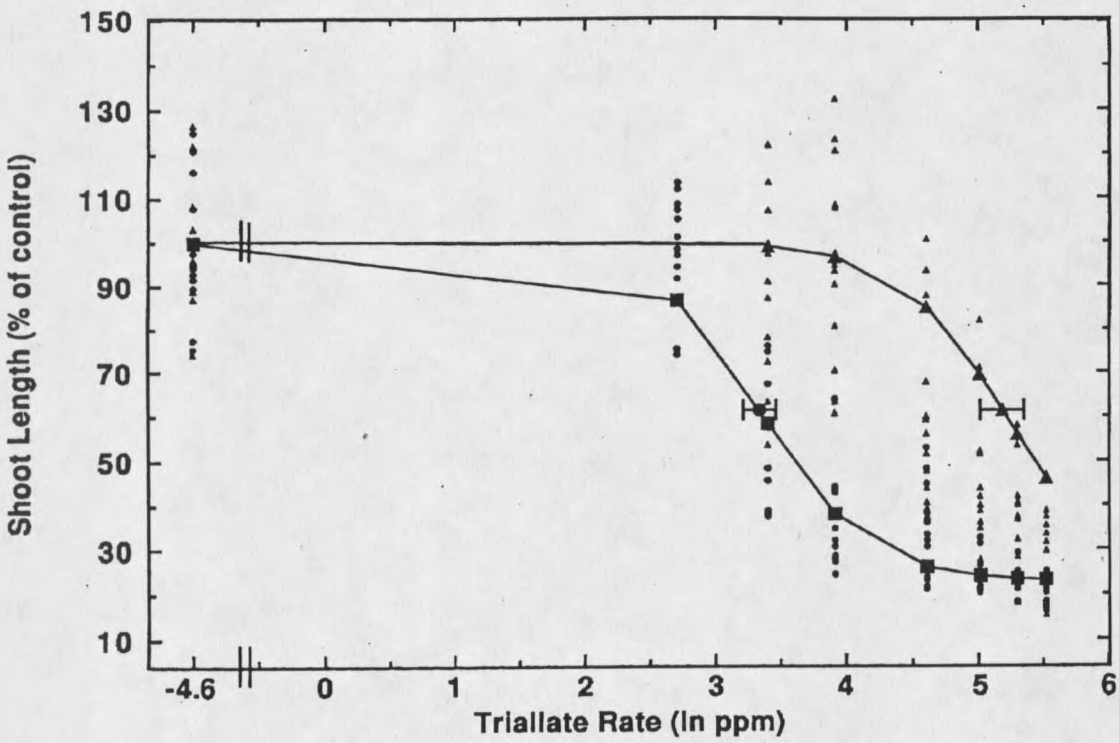

Figure 8. Growth inhibition of FG93R22 (triangles) and SH430 (circles) wild oat lines by triallate application in Petri dishes. Horizontal error bars represent the 95% confidence intervals of the natural log of triallate doses needed to inhibit growth by 50% (±3.3 ppm for SH430 and ±27.1 ppm for FG93R22; see Methods and Materials).

and 28 ppm, respectively (Figure 8), and the responses of FG93R22 and SH430 were representative of other R and S populations tested (data not shown). These values represent an approximate six-fold increase in triallate tolerance in R lines over S lines, compared with the 17-fold increase measured in the greenhouse dose response experiment. This decrease in observed resistance

levels may be due to the increased exposure to liquid and vapor-phase triallate in the sealed Petri dish system, as compared to more limited exposure in triallate-treated soil.

O'Donovan (1995) and Blackshaw et al. (199_) showed that triallate-resistant wild oat lines from Alberta were not cross-resistant to two other thiocarbamates and 17 herbicides from other families. However, R lines had not been previously tested for their sensitivity to diallate, a closely related but discontinued thiocarbamate herbicide. Therefore, FG93R22 and other R lines were treated with diallate in Petri dish assays as described for triallate. S lines were extremely sensitive to diallate inhibition, with estimated GR_{50} values of about 26 ppm (Figure 9). In contrast, the GR_{50} value for FG93R22 was estimated at about 215 ppm, representing more than an eight-fold increase in diallate resistance levels. The levels of sensitivity exhibited by the R and S lines shown in Figure 9 were representative of other R and S lines tested (data not shown). Because diallate was not tested in greenhouse dose response experiments the R/S GR_{50} ratio is not known for greenhouse conditions. All triallate-resistant lines tested were cross-resistant to diallate.

Triallate Sulfoxide Dose Response.

Previous reports (Casida et al., 1979, Lay & Casida, 1983) reported that the sulfoxide derivatives of pebulate and butylate were slightly more phytotoxic than the parent molecules, and the authors proposed these derivatives were the

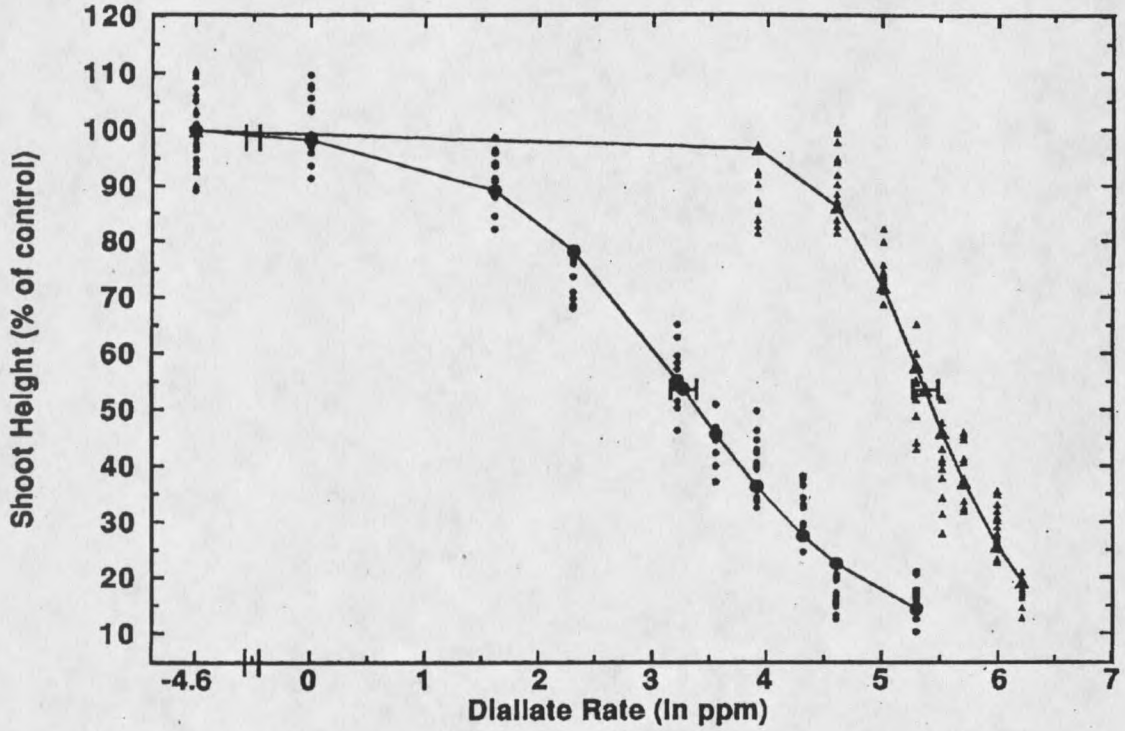

Figure 9. Growth inhibition of FG93R22 (triangles) and SH430 (circles) wild oat lines by diallate application in Petri dishes. Horizontal error bars represent the 95% confidence intervals of the natural log of diallate doses needed to inhibit growth by 50% (±2.8 ppm for SH430 and ±22.2 ppm for FG93R22; see Methods and Materials).

herbicidally active compounds. However, because of poor stability of the sulfoxide derivatives of the S-chloroallyl thiocarbamates such as diallate and triallate, this hypothesis had not been adequately addressed in *in vivo* plant studies. Therefore, dose response studies were carried out to compare phytotoxicity of triallate sulfoxide with triallate on R and S lines.

Because previous reports have shown that the sulfoxide derivatives of the

S-chloroallyl thiocarbamates have half lives of less than ten minutes in powder form (Schuphan et al., 1979), application of 1-μl treatment solutions of triallate sulfoxide would likely cause rapid evaporation of the solvent and subsequently degradation of triallate sulfoxide. Therefore, treatments were conducted using the root uptake method described in Methods and Materials, which would allow the triallate sulfoxide to remain in solution and slow degradation. Using this method, triallate sulfoxide had an estimated half-life of 4 hours at room temperature (data not shown).

Preliminary evidence (Colliver et al., 1994) indicated that triallate resistance in wild oats was due to decreased herbicide metabolism. If triallate sulfoxide was not being produced in R plants, externally applied triallate sulfoxide should be equally phytotoxic to both R and S wild oats. In addition, comparison of the phytotoxicity of triallate sulfoxide to triallate would provide evidence whether triallate sulfoxide is a herbicidally active compound. Due to the altered treatment methodologies, direct comparisons between the phytotoxicity of triallate as applied previously in Petri dish dose response assays and triallate sulfoxide could not be made. Therefore, concurrent tests using the root uptake method were done with R and S wild oats with both triallate and the triallate sulfoxide.

Triallate sulfoxide was slightly more phytotoxic than triallate to SH430 seedlings in this system, with an estimated GR_{50} value of six ppm for triallate sulfoxide and 17 ppm for triallate in the Petri dish assay (Figure 10). For R seedlings, GR_{50} values for triallate and triallate sulfoxide were estimated at 150

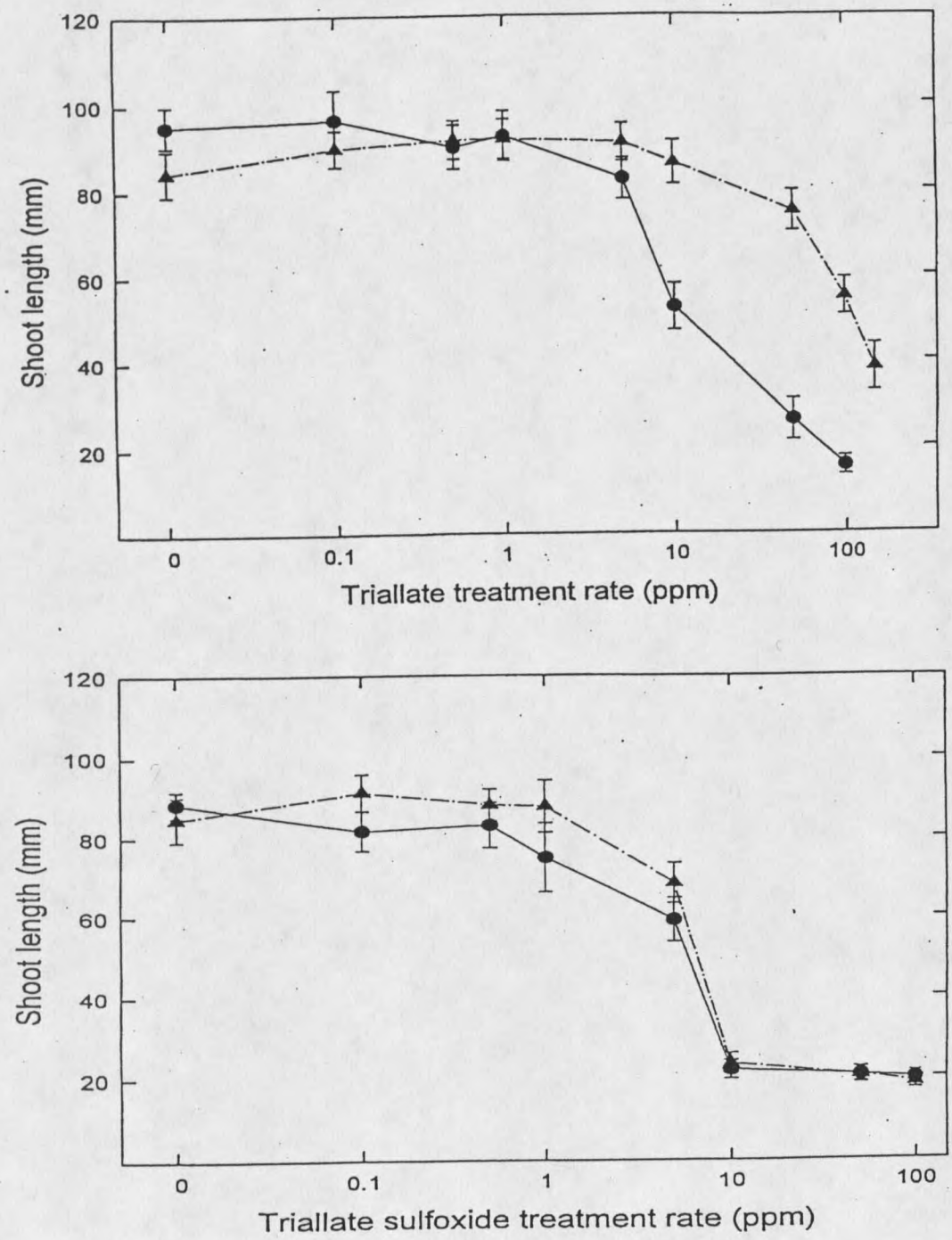

Figure 10. Growth inhibition of FG93R22 (triangles) and SH430 (circles) wild oat lines by root uptake of triallate (top) and triallate sulfoxide (bottom) in Petri dishes. Vertical error bars represent the standard error of the means at each treatment rate.

and seven ppm, respectively. Thus, even though R seedlings were about 9-fold more tolerant to triallate treatment than S seedlings in this experimental system, R and S seedlings were equally sensitive to triallate sulfoxide. These data support the idea that triallate sulfoxide is more phytotoxic than triallate. In addition, the observation that R and S seedlings were equally sensitive to triallate sulfoxide may provide an important insight into a probable resistance mechanism. The most logical explanation for these results is that R wild oats are deficient in triallate sulfoxidation. Further support and discussion of this hypothesis continues on page 43.

Triallate Metabolism

^{14}C-labeled triallate and technical grade triallate were used to determine and quantify herbicide metabolic profiles in R and S wild oats. Suitable chromatographic conditions were developed to separate triallate (retention time 17.0 minutes in this chromatography system) from metabolites generated *in vivo* (Figure 11). Based on cochromatography with radiolabeled triallate under the HPLC conditions of Figure 11, Peak IV was identified as unmetabolized triallate. S and R wild oat seedlings treated with ^{14}C-labelled triallate metabolize triallate (Peak IV) extensively, forming one major metabolite (Peak I) and two minor metabolites (Peaks II and III) under our experimental conditions. Metabolites formed in both R and S wild oats had identical retention times.

Peak I in Figure 11 eluted at 2.1 minutes, the void volume under these

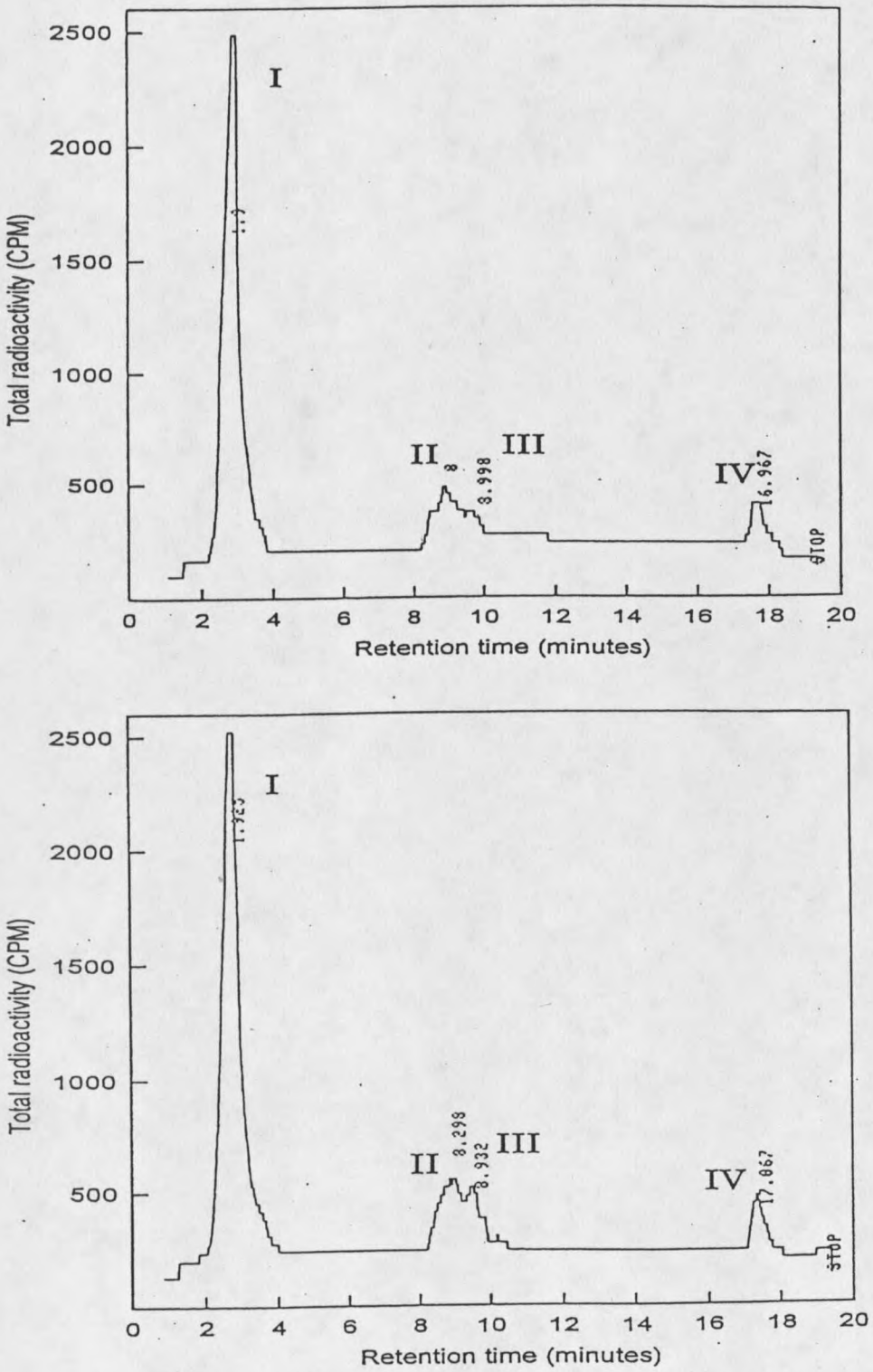

Figure 11. Representative HPLC chromatograms of ^{14}C-labelled triallate and metabolites eluted using reverse-phase HPLC. S (top) and R (bottom) wild oat plants were harvested 24 and 168 hours after treatment, respectively.

conditions, prompting me to consider the possibility that it may have been trichloropropene sulfinic acid (TCPSA), a triallate metabolite identified in *Pisum sativum* (Hackett et al., 1994). Because of the highly polar nature of this compound, it would not be expected to be retained using C18 reverse phase protocols. A Weak Anion Exchange (WAX) protocol was developed as outlined in Methods and Materials with the kind assistance of Kevin Young to better characterize Peak I and compare it with TCPSA.

Peak I was collected from fractions collected 2.0 to 2.2 minutes after injection in the C18 system and subjected to WAX chromatography. Retention times for both compounds were identical (Figure 12). In addition, Peak I was spiked with 7,000 dpm TCPSA, resulting in a quantitative increase in peak area when rechromatographed (data not shown). Therefore, Peak I has been identified as TCPSA. For a possible *in vivo* metabolic scheme, see Figure 13.

Peak IV was collected from fractions eluted 16.9 to 17.1 minutes after injection in the C18 system and re-chromatographed in a 65:35 acetonitrile:water isocratic mobile phase under otherwise identical HPLC conditions. Peak IV coeluted with the radiolabeled triallate standard at 6.6 minutes (data not shown). Efforts are now underway to identify Peaks II and III using a combination of HPLC purification and mass spectroscopy (MS) and gas chromatography/MS.

The first characterizations of triallate metabolic patterns in R and S wild oats indicated that both types metabolized triallate to the same products (Figure 11). However, rates of triallate metabolism in R and S wild oats appeared to be quite different. Therefore, R and S wild oat seedlings were treated with

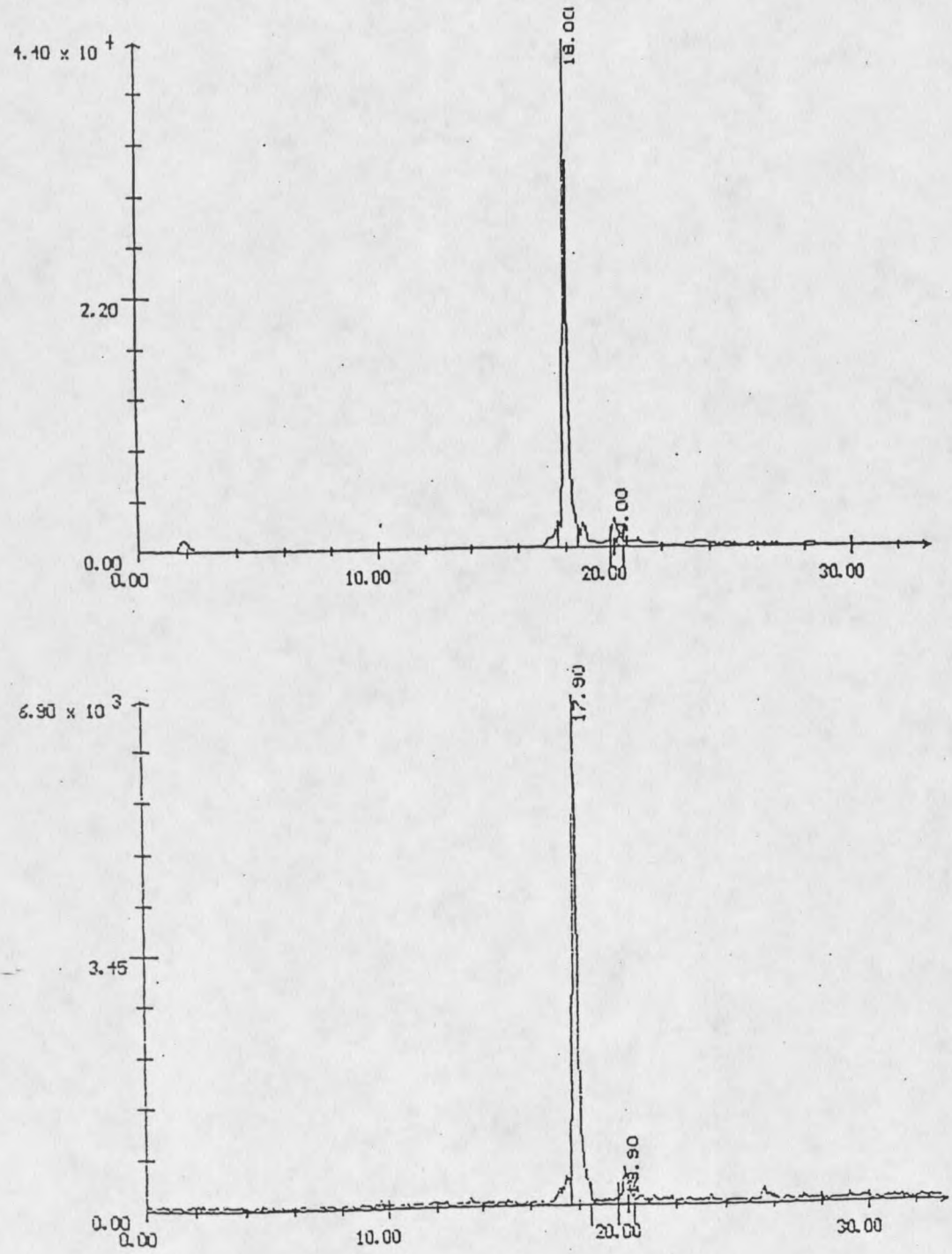

Figure 12. HPLC chromatograms of Peak I from Figure 11 (top) and TCPSA standard (bottom) in the WAX chromatography system. X axes are retention times in minutes; Y axes are total radioactivity measured in counts per minute.

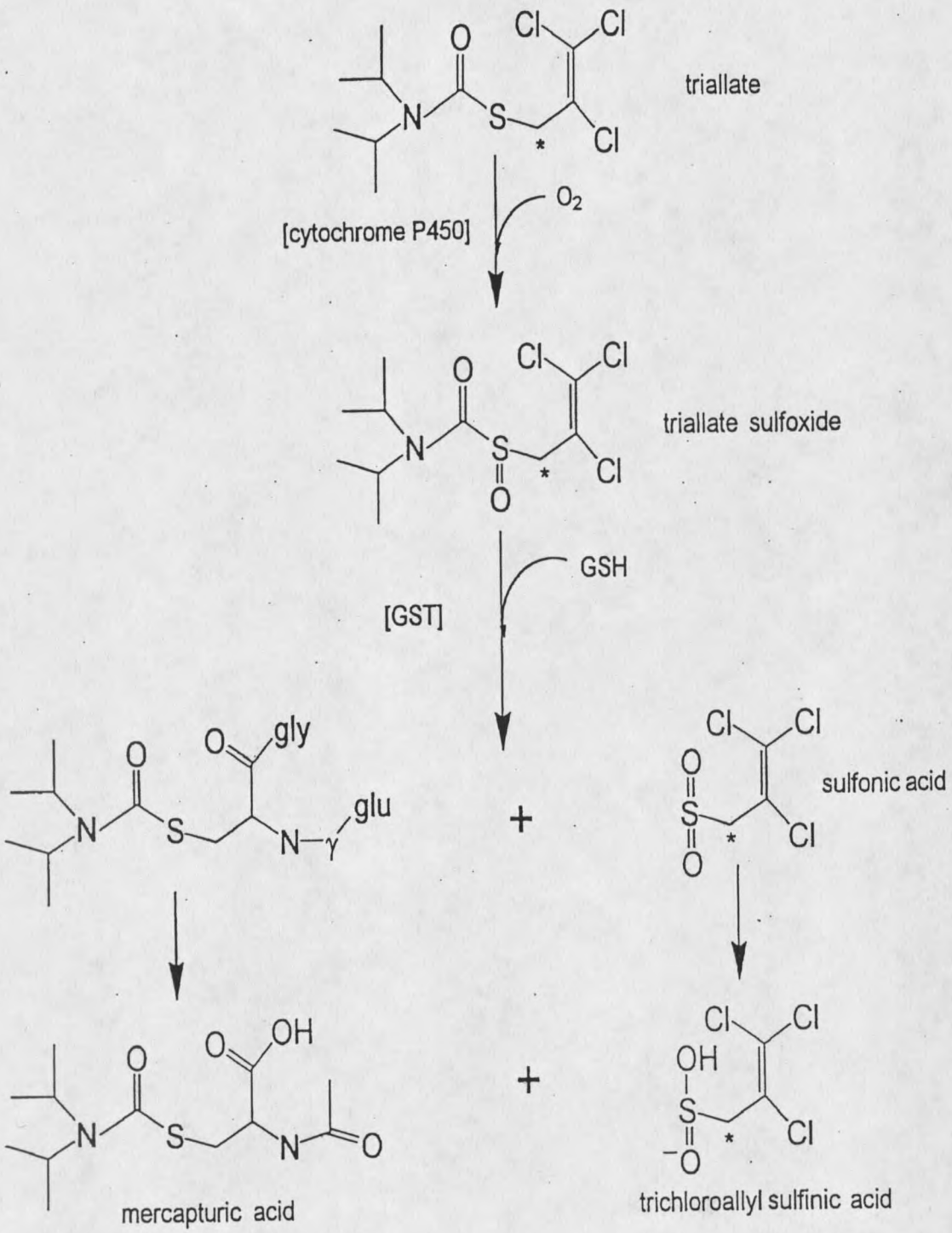

Figure 13. Proposed primary pathway of triallate metabolism in R and S wild oats, including enzymes and cofactors. Partially modified from Hackett et al. (1994) and Schuphan et al. (1979). Position of radiolabel indicated by asterisks.

^{14}C-labelled triallate and harvested after 1, 3, 6, 9, 12, 24, 36, 48, 60, 72, or 96 hours and extracted as described above. After standardization of radioactivity present in samples as outlined in Materials and Methods, extracts were subjected to HPLC analysis as described above.

Representative chromatograms of extracts from R and S wild oats 24 hours after treatment are shown in Figure 14. Triallate was metabolized much more slowly in R than S seedlings, as indicated by the slower disappearance of triallate and the slower appearance of metabolites in R biotypes. To determine the rate of triallate metabolism in each biotype, integrated peak area values for triallate and its metabolites from the treatments described above were compared between R and S seedlings (Table 1).

In S plants, more than 50% of the applied triallate was metabolized into TCPSA and other compounds by 9 hours after treatment, with nearly total disappearance of triallate after 48 hours. In contrast, about 50% of the applied triallate remained unmetabolized in R biotypes by 96 hours after treatment. Half-life values for triallate in S and R wild oats estimated from these data were 8 hours and greater than 96 hours, respectively. Thus, although it appears that the R plants are producing the same metabolites as S, the rate of triallate metabolism is more than ten-fold slower.

Based on these data, I hypothesize that R plants contain a mutation that confers a significantly reduced rate of triallate sulfoxidation (activation). If the formation of triallate sulfoxide is required for herbicidal activity, then slower rates of sulfoxidation would reduce the effective herbicide concentrations *in vivo*, and

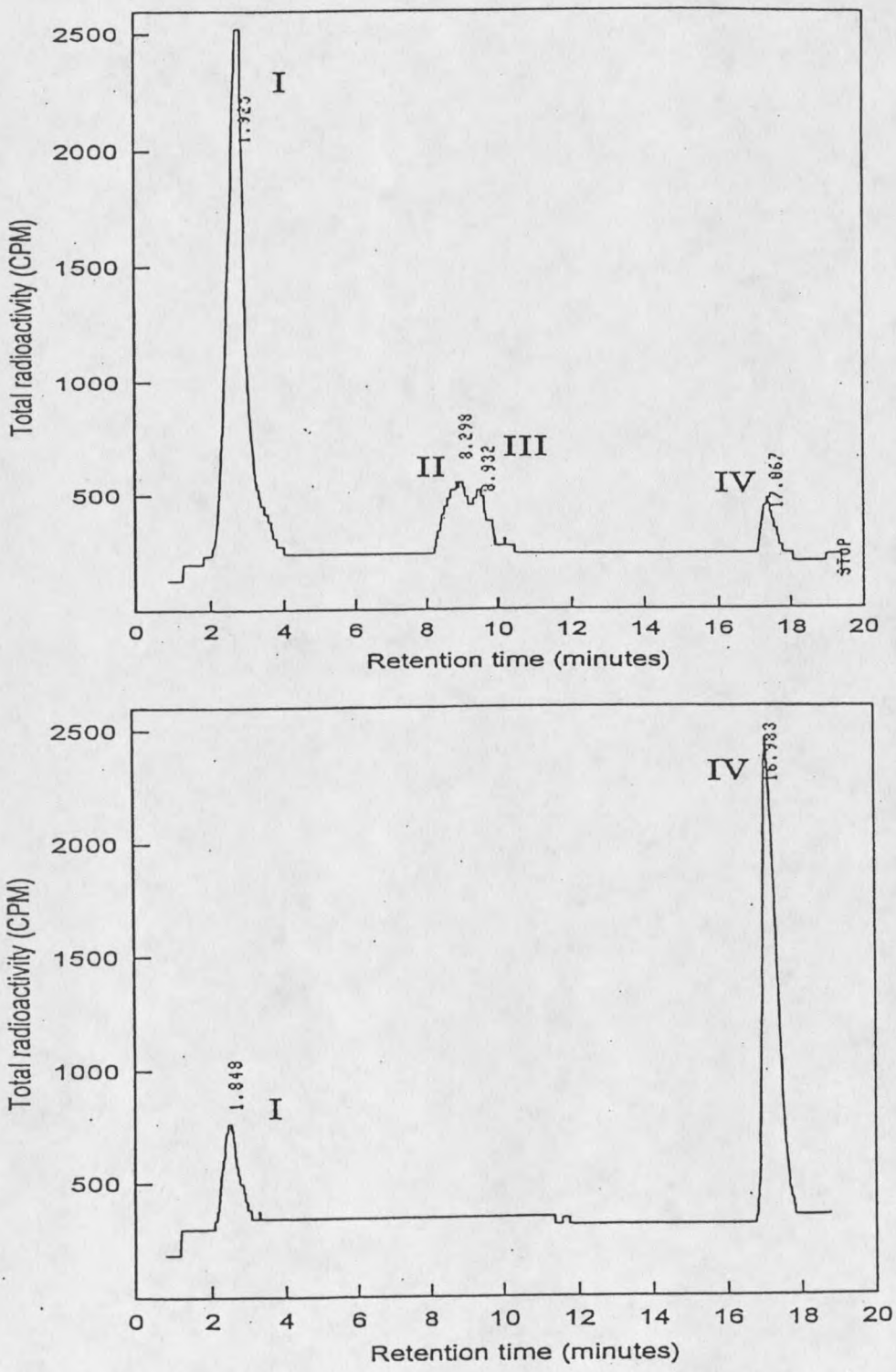

Figure 14. Representative HPLC chromatograms from S (top) and R (bottom) wild oats 24 hours after treatment with ^{14}C-labelled triallate.

Time after treatment (h)	SH430 Triallate	SH430 TCPSA	SH430 Other	FG93R22 Triallate	FG93R22 TCPSA	FG93R22 Other
1	90 (3.1)	2 (1)	8 (1.1)	100 (0)	0 (0)	0 (0)
3	81 (4.4)	8 (2.2)	11 (2.4)	99 (1.3)	0 (0.1)	1 (0.2)
6	63 (4.5)	20 (4.4)	16 (4)	98 (1.3)	1 (0)	1 (0)
9	44 (4.2)	41 (3.3)	15 (3)	98 (2.6)	0 (0.2)	1 (0.2)
12	19 (3.3)	61 (3.8)	19 (3.1)	92 (3.2)	2 (0.1)	6 (0.7)
24	7 (3.5)	82 (2)	11 (2.2)	87 (2.8)	4 (0.3)	9 (0.6)
36	5 (2.2)	85 (5.5)	11 (1.7)	76 (8.2)	10 (1.7)	13 (2.5)
48	2 (1.1)	80 (8.2)	18 (6.6)	67 (2.9)	22 (3.3)	11 (2.1)
60	1 (0.7)	92 (4.1)	7 (2.4)	69 (9.3)	26 (5)	5 (3.9)
72	0 (0)	98 (3)	2 (0.3)	55 (3.6)	33 (4.6)	12 (3.4)
96	0 (0)	91 (1.6)	9 (0.8)	52 (5.6)	43 (4.8)	6 (1.1)

Table 1. Distribution of ^{14}C triallate and its metabolites in R and S wild oat seedlings at various times after treatment. Values are expressed as a percent of total radioactivity for triallate, TCPCA, and other metabolites (Peaks II and III). Values in parentheses are standard errors of the means.

subsequently fewer metabolites would accumulate. Less conversion of triallate to metabolites in R plants could also explain the reduced triallate uptake rates seen in R lines (Colliver et al. 1993), because of a reduced triallate concentration gradient across the plasmalemma. These data provide preliminary evidence for

the first example of resistance development due to decreased metabolism of a proherbicide.

CHAPTER 3

INVESTIGATIONS INTO THE MECHANISM OF DIFENZOQUAT RESISTANCE IN WILD OATS

In response to field reports of poor triallate performance beginning in 1991 and 1992, O'Donovan et al. (1994) investigated the response of several Canadian wild oat populations to triallate and other herbicides. The authors showed that triallate-resistant wild oats were also cross-resistant to difenzoquat. At about the same time, wild oat field collections from near Fairfield, MT were tested due to producer complaints of non-performance. Malchow et al. (1993) reported that 61% of the fields sampled contained triallate-resistant wild oats. Some of these populations were later tested and were shown to be cross-resistant to difenzoquat (Kern et al., 1994; Malchow, 1995). Because of the limited number of herbicides commercially available for wild oat control in small grain crops, the loss of two economically important herbicides presents a serious concern for small grain producers. Therefore, I began studies to characterize and compare the physiological and biochemical fate of difenzoquat in R and S wild oats to gain insights into the mechanism of difenzoquat resistance in wild oats.

Methods and Materials

Biotype Selection

S wild oat seeds used for all experiments were collected from populations of the nondormant inbred line SH430 (Jana and Naylor, 1976) grown at the Arthur H. Post Research Farm near Bozeman, MT in 1993. R seed used for some greenhouse dose response experiments was collected from fields near Fairfield, MT in which triallate had been used annually for several years (Malchow et al., 1993). Seeds were collected from plants that had survived triallate treatment the preceeding spring and that were shown to be resistant to triallate (Chapter 2) and difenzoquat. For uptake, translocation, and metabolism experiments, one difenzoquat-resistant field collection (FG93R28) and an inbred triallate- and difenzoquat-resistant line (FG93R22; see Chapter 2 for lineage development) were used.

Greenhouse Dose Response

Three rows of 12 caryopses each from lots SH430, FG93R28, two S field accessions, and nine other triallate-resistant field collections were planted in 55 x 35 x 10 cm flats in greenhouse soil mix (1:1:1 Bozeman silt loam:washed sand:peat moss (v/v/v)), watered as needed, and fertilized[9] weekly. Flats were held in the greenhouse with a 14-hr daylength under natural sunlight

[9]Peter's 20:20:20. Grace Sierra Horticultural Products Co., Milpitas, CA 95035.

supplemented with mercury vapor lamps and day/night temperatures of 22/16° C. After 21 days, four leaf-stage plants were selected for uniformity and 15 to 20 plants for each treatment were treated with 0, 0.084, 0.21, 0.63, 0.84, 1.26, 1.68, or 3.36 kg/ha difenzoquat (for S plants) or 0, 0.84, 1.68, 3.36, 5.04, 6.72, 8.4, 13.44, or 20.16 kg/ha difenzoquat (for R plants) in a volume of 194 L/ha water containing 0.25% nonionic surfactant[10] using a moving belt sprayer. Herbicide solutions were allowed to dry, and the plants were returned to the greenhouse conditions described above, fertilized weekly, and watered as needed. After 30 days, shoots were harvested by clipping at the soil surface, and individual fresh weights determined. There were 3 replications of five to seven plants each for all treatments and the experiment was repeated once.

Dose Response Statistical Analysis

Data analysis was conducted by Mr. Corey Colliver using programs written by Dr. Bruce Maxwell, Department of Plant, Soil, and Environmental Sciences, Montana State University-Bozeman as described in Chapter 2 (page 28). For the difenzoquat models the curves were not parallel.

Difenzoquat Uptake

FG93R22 and SH430 seedlings were grown in the greenhouse as described above until the four-leaf stage. A 2-cm section of the third leaf (1 cm

[10]X-77 nonionic surfactant. Valent USA Corp., North Carolina Blvd., Walnut Creek, CA 94596.

acropetal to the ligule) was covered with aluminum foil and the plants were treated with 0.84 kg/ha formulated difenzoquat as described above. After drying, the foil was removed and 1.67 x 10^3 Bq 3-^{14}C-labelled difenzoquat (specific activity 2.29 x 10^6 Bq/mg) was applied in five 1-ul droplets on the unsprayed 2-cm leaf section using a Hamilton syringe. Treatment solutions were allowed to dry and the flats were placed in a growth chamber under a 14-hour daylength and day/night temperatures of 22/18° C. To determine uptake, the treated leaf section was excised 12, 24, 48, 72, or 96 hours after treatment, rinsed in 3 ml water containing 0.25% surfactant for 10 minutes and oxidized[11]. Radioactivity from evolved $^{14}CO_2$ and ^{14}C present in a 0.25 ml aliquot of the leaf rinsate were quantified by liquid scintillation counting. Percent uptake was determined by subtracting the radioactivity present in the leaf rinsate from the total applied. Total recovery (±5%) of radioactivity was verified by summing radioactivity in the leaf with the radioactivity recovered by sample oxidation. There were six replicates for each treatment in a randomized complete block design, and the experiment was repeated once.

Difenzoquat Translocation

To determine translocation, R and S plants were treated with 0.84 kg/ha difenzoquat and ^{14}C-labelled difenzoquat applied to the third leaf as described above. Plants were harvested 12, 24, 48, 72, or 96 hours after treatment and

[11]OX-300 Biological Sample Oxidizer, R.J. Harvey Instrument Corp., Hillsdale, N.J. 07642.

dissected into: 1) the 2-cm treated leaf section (washed as above to remove any unabsorbed radioactivity), 2) the leaf tissue acropetal to the treated section, 3) the leaf tissue basipetal to the treated section, including the leaf sheath, 4) the meristematic region, defined as the region of stem from root emergence to 1.5 cm above the soil surface, and 5) the remaining portion of the plant, including roots. The samples were oxidized and ^{14}C quantified as described above. There were eight replicates for each treatment in a randomized complete block design, and the experiment was repeated twice.

Difenzoquat Metabolism

R and S seeds were grown in the greenhouse and four leaf-stage plants treated with 0.84 kg/ha formulated difenzoquat as described above. Plants were treated by spotting 1.67×10^3 Bq 3-^{14}C-labelled difenzoquat on the third leaf as described above. After 12, 24, 48, 72 or 96 hours, the treated leaf was excised and rinsed as described above to remove unabsorbed difenzoquat. A 3-cm section of leaf immediately acropetal to the treated section was excised and homogenized in a Broeck ground glass homogenizer containing 0.5 ml water with 0.25% surfactant[10]. The homogenate was centrifuged at 13,600 x g for 10 minutes at room temperature and the supernatant removed and filtered through a 0.22 micron nylon centrifuge filter. The pellet was recovered, re-homogenized in 0.5 ml 100% ethanol, the homogenate centrifuged as above, and the resulting supernatant was removed and filtered as above. The resulting pellet was again

homogenized in 0.5 ml 100% benzene, the homogenate centrifuged, and the supernatant removed and filtered as above. The final pellet was ozidized to determine unextractable radioactivity. All supernatants from the serial extractions were then standardized to 25,000 dpm by dilution with the appropriate solvent and subjected to HPLC analysis.

HPLC conditions for difenzoquat metabolism analysis used a C18 reverse phase column in 4.6mm x 250mm format as outlined in Chapter 2. The mobile phase consisted of 55% acetonitrile:45% 0.5% sodium perchlorate. The HPLC pump delivered solvent at 1.5 ml/minute and radioactive compounds were quantified using an in-line scintillation detector. Under these conditions, difenzoquat eluted as a single peak at 5.5 minutes.

Results and Discussion

Difenzoquat Dose Response Experiments

All triallate-resistant wild oat lines tested from Alberta were highly cross-resistant to difenzoquat (O'Donovan et al., 1994). Similarly, some R lines tested from collections near Fairfield, MT were also resistant to difenzoquat (Kern et al., 1994), although Malchow (1995) reported that only 43% of the 23 lines tested were cross-resistant to difenzoquat in a field study. The inbred S line SH430 and other susceptible lines were very sensitive to difenzoquat treatment. Thirty days after treatment, characteristic injury symptoms were present at doses above 0.43

kg/ha, including profuse tillering, severe stunting, and a characteristic dark green color. GR_{50} values for S lines were estimated to be about 0.18 kg/ha difenzoquat, while R GR_{50} values were greater than 12 kg/ha difenzoquat, indicating an approximate 65-fold increase in resistance levels (Figure 15).

These GR_{50} values for R and S wild oats generally agree with the findings of O'Donovan et al. (1994), who reported a 10- to 100-fold increase in difenzoquat tolerance in R wild oats, although results varied with repetitions. Accurate GR_{50} values could not be directly measured for some R lines tested because growth of these lines was not inhibited by any difenzoquat dose tested, including treatment with undiluted formulated difenzoquat applied to the point of run-off from the plant (about 80 kg/ha; data not shown).

Difenzoquat Uptake

^{14}C-labelled difenzoquat was taken up rapidly in both R and S plants for the first 24 hours after application (Figure 16). About 50% of the applied radioactivity was absorbed within 24 hours in S leaves, and reached a maximum of about 70% absorption by 96 hours after application. ^{14}C-difenzoquat uptake was similar in R and S lines by 12 hours after application, but uptake was consistently about 15 to 20% higher in R plants at the remaining time points. Although these differences are significant, it seems unlikely that resistance is caused by increased herbicide uptake in R plants. Overall, rates of difenzoquat uptake in both lines were similar to those reported by Sharma et al. (1976), but

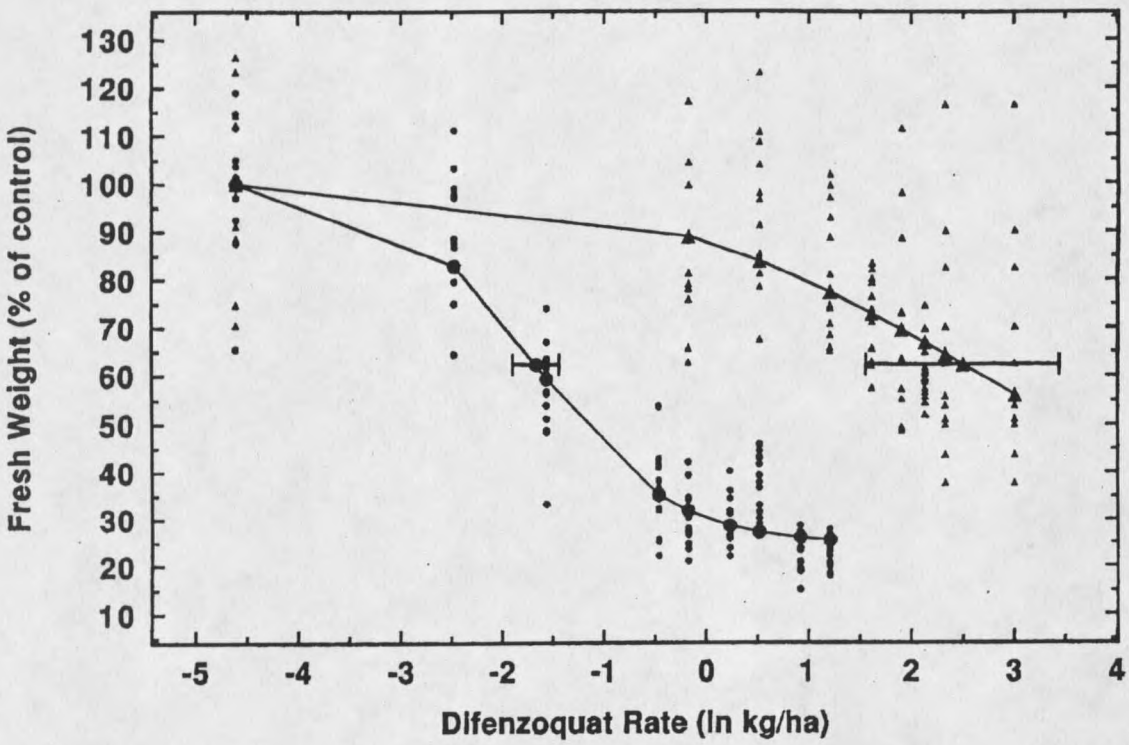

Figure 15. Growth inhibition of FG93R22 (triangles) and SH430 (circles) wild oat lines by greenhouse difenzoquat treatment. Horizontal error bars represent the 95% confidence intervals of the natual log of difenzoquat treatment rates needed to inhibit growth of each biotype by 50% (±0.39 kg/ha for SH430 and ±7.43 kg/ha for FG93R22; see Methods and Materials).

were slower than those observed for other bipyridilium herbicides in other species (Brian et al., 1967).

Difenzoquat Translocation

^{14}C-difenzoquat translocation patterns were similar between R and S lines up to 12 hours after application (Table 2). However, after 12 hours a greater

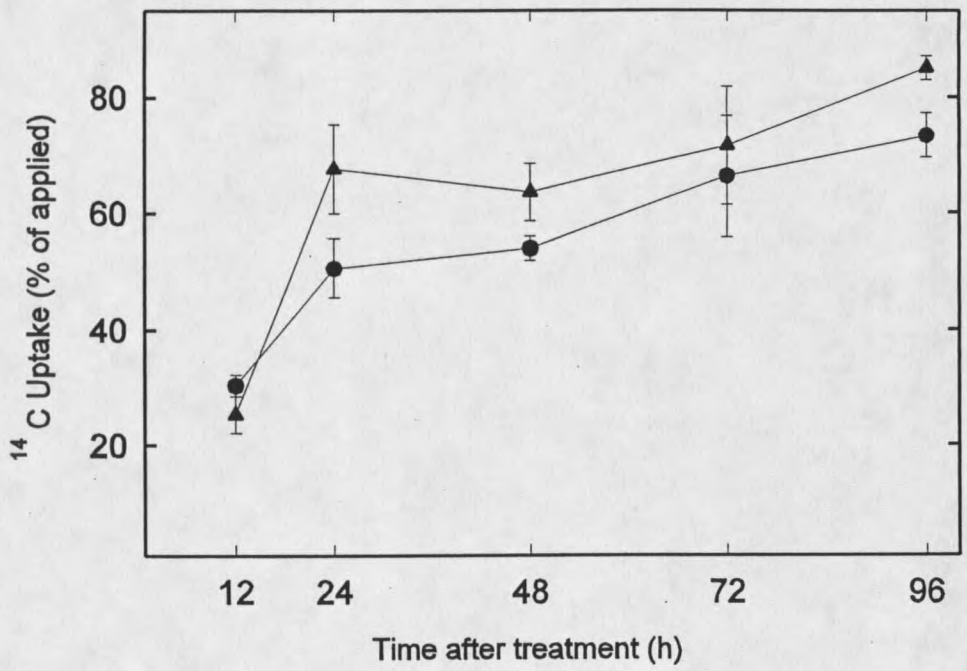

Figure 16. Uptake of ^{14}C-labelled difenzoquat in FG93R22 (triangles) and SH430 (circles) wild oats. Vertical bars represent standard errors of the means.

percent of the absorbed ^{14}C-difenzoquat was translocated away from the 2-cm treated section of leaf tissue in R plants than in S plants, as indicated by significantly lower ^{14}C levels in the treated section of R leaves. The increased amounts of ^{14}C translocated away from the treated zone in R plants appeared to be uniformly spread throughout the remainder of the treated leaf and the plant, although differences between R and S samples were not significant. Of the radioactivity absorbed in R and S leaves, about 15% to 30% (depending on the timepoint) was translocated to the leaf tissue acropetal to the treated spot, and

Tissue		Time after Application (h) 12	24	48	72	96
Treated section	(S)	77.7 (9.3)	58.1 (5.1)*	56.6 (4.3)*	48.6 (4)*	44.6 (4.8)*
	(R)	71.4 (5.2)	48.4 (4.2)	49.4 (4.2)	38.8 (5.7)	30.7 (6.5)
Leaf acropetal	(S)	12.3 (2.3)	19.8 (4.3)	16.6 (5.3)	21.9 (3.1)	23 (2.1)
	(R)	15.4 (2.1)	23.9 (5.1)	19.9 (3.8)	24.4 (2.3)	27.7 (3.3)
Leaf basipetal	(S)	3.1 (0.4)	6.8 (3.1)	8.2 (2.8)	8.7 (4.4)	10.2 (3.2)*
	(R)	5.3 (2.1)	9.1 (2.3)	12.2 (3.6)	13.6 (2.1)	16.5 (4.1)
Remainder of plant	(S)	6.4 (2.2)	13.1 (1.7)	14.1 (1.2)	16.3 (4.6)	17.8 (4.2)
	(R)	7.8 (1.6)	15.4 (2.4)	13.4 (2.7)	18.5 (3.6)	19.6 (3.1)
Meristem	(S)	0.1 (0.1)	2.4 (0.2)	4.9 (1.2)	4.6 (0.5)	4.2 (1.2)
	(R)	0.1 (0.1)	3.1 (0.3)	4.8 (0.8)	4.4 (0.7)	5.7 (1.1)

Table 2. Translocation of ^{14}C-difenzoquat in FG93R22 (R) and SH430 (S) wild oat tissues 12, 24, 48, 72, and 96 hours after application. Data are expressed as a percentage of the total radioactivity recovered from the plant at each timepoint. Values in parentheses are standard errors of the means. Values followed by an asterisk indicate significant differences between R and S within a tissue and harvest time.

about 5% to 15% was translocated basipetally in the treated leaf and leaf sheath. Only about 5% of the absorbed radioactivity was translocated to the meristematic tissue by 96 hours. These values are similar to translocation amounts reported by Sharma et al. (1976).

The minor alterations in difenzoquat uptake and translocation patterns observed in these studies do not appear to be sufficient to explain the dramatic resistance levels observed in the R wild oat lines. Because difenzoquat appears to have herbicidal activities both on meristematic growth as well as contact activities commonly associated with other bipyridilium herbicides (Shaner, 1983), it could be argued that an alteration in herbicide uptake, translocation or metabolism would be more likely to be selected for in the field than an alteration in the site(s) of action.

Difenzoquat Metabolism

HPLC analysis of extracts from ^{14}C-difenzoquat treated R and S plants showed that all radioactivity eluted at 5.5 minutes, the retention time for difenzoquat, and no radioactive metabolites were detected at any time point (data not shown). In addition, more than 90% of the recoverable radioactivity was found in the water extracts from R and S leaf tissues. Further extraction of pellets with ethanol and benzene produced only trace amounts of radioactivity (data not shown). However, in comparing the amounts of water-extractable radioactivity in R and S leaf tissues over time, significant differences were observed (Table 3). In S plants, the total amount of radioactivity in leaf tissues

Time after treatment (h)	SH430 Total	SH430 Extractable	SH430 Percent extractable	FG93R22 Total	FG93R22 Extractable	FG93R22 Percent extractable
12	12,100	11,800	97.5 (8.4)	14,400	8,200	56.9 (8.1)
24	18,700	17,900	95.7 (6.6)	24,000	7,300	30.4 (5.5)
48	20,400	18,900	92.6 (10.4)	27,000	5000	18.5 (7.8)
72	21,600	20,400	94.4 (4.9)	31,400	3,100	9.8 (3.4)
96	23,100	22,200	96.2 (7.2)	33,100	2,200	6.6 (6.2)

Table 3. Total and extractable radioactivity in R and S wild oats at various times after treatment with ^{14}C-difenzoquat. Values in parentheses are the standard errors of the means.

(as determined by sample oxidation) increased from 12,100 cpm to 23,100 cpm between 12 and 96 hours after treatment. Of these amounts, more than 90% was recoverable in aqueous extracts, and the percent recovery did not change over time. In R plants, total radioactivity increased from 14,400 cpm to 33,100 cpm between 12 and 96 hours after treatment, most likely as a result of increased translocation as shown in Table 2. In contrast to recovery from S

leaves, percent extractable radioactivity in R leaves was only 57% after 12 hours, and recovery steadily decreased to only 7% by 96 hours after treatment. The fact that this amount of radioactivity was not extractable in ethanol or benzene indicates that the herbicide molecule had been altered and bound to an insoluble cellular component. Further experimentation is underway to determine the nature of this alteration and the ultimate fate of the difenzoquat molecule in R plant tissues.

Although there are no reports of difenzoquat metabolism in any plant species tested, I hypothesize that the slightly increased difenzoquat uptake (Figure 16) and translocation rates (Table 2) observed in R plants may indicate the presence of a novel metabolizing activity. If difenzoquat is being bound into extracellular material, R plants would be injured less than S plants. This decreased injury would allow continued xylem transport of solutes (including difenzoquat), thus increasing difenzoquat translocation. In addition, the apparently irreversible binding of difenzoquat, perhaps in cell wall material, further supports the hypothesis that resistance in R wild oats is conferred by a unique difenzoquat metabolizing pathway that very effectively immobilizes difenzoquat present in the cell matrix, perhaps even before it crosses the plasmalemma. Powles and Cornic (1987) showed that *H. glaucum* biotypes resistant to paraquat had decreased cellular uptake of paraquat, resulting in non-enzymatic sequestration of paraquat into cell walls. In addition, Hart et al. (1992) showed that more than 75% of applied paraquat became irreversibly associated with maize root cell walls within 24 hours after application. These studies

indicate that bipyridilium herbicides may have a propensity for binding to cell wall material. It is possible that difenzoquat resistance in wild oats results from a similar non-enzymatic sequestration.

CHAPTER 4

EFFECTS OF TRIALLATE AND TRIALLATE SULFOXIDE ON LIPID BIOSYNTHESIS IN WILD OATS

The mode of action of triallate and the thiocarbamates is not clearly understood. EPTC and other thiocarbamates have been shown to inhibit the formation of epicuticular waxes in certain species (Gentner, 1966; Fuerst, 1985), but definitive inhibition of specific enzymes has not been documented. Several studies showed that fatty acid elongases are inhibited by treatment with the thiocarbamates or their sulfoxide derivatives (Harwood et al. 1989; Harwood 1994). However, studies addressing the synthesis of long-chain fatty acids and their precursors have yielded conflicting results. Rajasekharan and Sastry (1989) reported that diallate inhibited ^{14}C-acetate incorporation into all polar and neutral lipid fractions tested; however, Ezra et al. (1983) showed that EPTC had no effect on ^{14}C-acetate incorporation into polar lipid fractions. Several authors (Abulnaja and Harwood, 1991; Barrett and Harwood, 1993; Bolton and Harwood, 1976) showed that thiocarbamates and thiocarbamate sulfoxides inhibit fatty acid elongation but not synthesis of 16- and 18-carbon fatty acids. However, the same authors (Abulnaja and Harwood, 1991) showed in whole plant studies diallate and triallate inhibited only fatty acid elongases, in contrast to inhibition of

all fatty acid synthesis in microsomal fractions from pea. Because of these discrepencies, the authors proposed that *S*-chloroallyl thiocarbamate sulfoxides (which are not formed in microsomal fractions) selectively inhibited fatty acid elongases without affecting synthesis of short-chain fatty acids. The objectives of these studies were to 1) determine the effect of triallate treatment on formation and deposition of epicuticular waxes in R and S wild oats; 2) determine the effect of triallate treatment on synthesis of nonpolar fatty acids in R and S wild oats; and 3) determine the effect of triallate sulfoxide treatment on synthesis of nonpolar fatty acids in R and S wild oats.

Methods and Materials

Epicuticular Wax Deposition

Caryopses from the inbred triallate-resistant line FG93R22 and the inbred susceptible line SH430 were used for all experiments (see Chapter 2 for lineage development). Caryopses were planted in greenhouse soil mix as described previously and grown under the conditions described above until seedlings were approximately 2 cm tall (about 7 days after planting). R and S seedlings were treated with 0.1 kg/ha triallate using a greenhouse belt sprayer as described above. Five days after treatment, R and S seedlings were about 4 cm tall, and a clear tissue constriction was visible on the shoot, marking the location of the shoot apex at the time of treatment. This visible damage allowed me to dissect

each seedling into tissue present before and after treatment, and quantify surface waxes from these tissues separately. After dissection, leaf areas were quantified using a leaf area meter[12].

Epicuticular waxes present on R and S seedlings before and after treatment were isolated by ten 1-second dips in 0.5 ml hexane. Samples were evaporated to dryness, resuspended in a minimal volume of HPLC grade hexane and derivatized using trimethylsilene. The derivatives were subjected to gas chromatography (GC) analysis, using a 12 m capillary column[13] with helium as a carrier gas. After sample injection, oven[14] temperature was ramped from 150°C to 300°C at 5°/minute, and eluted compounds detected with a flame ionization detector. Samples were quantified by integration[15] and identified by comparison with C26 authentic standards. There were 12 R and 12 S seedlings for the treatment, and the experiment was repeated once.

Analysis of Free Fatty Acids

To investigate the effect of triallate and triallate sulfoxide treatment on biosynthesis of short-, medium-, and long-chain fatty acids (C12 to C26), 1 μl of 10 or 100 ppm (v/v) triallate or 1 μl of 100 or 500 ppm (v/v) triallate sulfoxide (for synthesis, see Chapter 2) was spotted on the coleoptile tips of 4-day-old R

[12]LI-3100 Leaf Area Meter. Li-Cor, Inc., Lincoln, Ne 44103.
[13]HP-1 capillary column. Hewlett Packard, Inc., Wilmington, De 19808.
[14]HP 5890 Series II Gas Chromatograph. Hewlett Packard, Inc., Wilmington, De 19808.
[15]Hewlett Packard Model 3396 Series II Integrator. Hewlett Packard, Inc., Wilmington, De 19808.

and S etiolated seedlings as described previously. Plants were harvested after 48 hours and homogenized in a Broeck glass homogenizer containing 0.5 ml HPLC-grade methanol. After 3 minutes of homogenization, 1 ml HPLC-grade methylene chloride added and the mixture homogenized for an additional 1 minute. The homogenate was partitioned twice with 0.5 ml distilled deionized water, and the water/methanol layer removed. A 250 μl aliquot of the sample in methylene chloride was removed, filtered through a 0.22 μm nylon microspin centrifuge filter. The samples were derivatized as above and subjected to GC analysis using an HP Series II Plus gas chromatograph. Separation of fatty acids was achieved using an Alltech Carbowax capillary column in 30 m format using hydrogen as carrier gas. Trimethylsilyl derivatives of isolated fatty acids were detected using a flame ionization detector using water-free air as makeup gas for a total flow rate of 400 ml/min. Fatty acids were identified based on coelution with known standards, and quantified with a Hewlett Packard model 3396 Series II integrator. There were 8 R and 8 S seedlings for the treatment, and the experiment was repeated once.

Results and Discussion

Epicuticular Wax Deposition

Wild oat seedlings were more tolerant to post-emergence applications of triallate than to preemergence applications, and 0.1 kg/ha triallate proved to be

the minimum sub-lethal dose required for visible S plant injury symptoms. Two days after treatment, a visible pinching effect was visible on the seedlings, which marked the location of the meristematic region at the time of application. Epicuticular wax deposition was visibly inhibited in S plants after application, noted by a marked dulling and deep blue color of the tissues which grew after triallate application.

The visible reduction in epicuticular wax deposition was verified by gas chromatographic analysis (Figure 17). The major wax component (95%) of untreated R and S seedlings was determined to be a C26 primary alcohol by mass spectroscopy (data not shown) as reported by Whitehouse et al. (1988), with typical concentrations of about 33 $\mu g/cm^2$. Five days after treatment, wax deposition in S wild oats was inhibited by over 80%; however, wax deposition in R wild oats was not affected. Inhibition of epicuticular wax deposition in S wild oats continued to be reduced up to 21 days after treatment with no effect on R plants. Wax constituents were not affected in either R or S wild oats by triallate treatment.

These data confirm the findings of Gentner (1966) and Harwood et al. (1989), who reported a dramatic reduction in the deposition of epicuticular waxes after triallate treatment of susceptible species. However, deposition of the C26 alcohol was not affected in R plants. Because of the lack of visual plant injury symptoms at this treatment rate, it is not surprising that R plants showed no reduction in epicuticular wax deposition. If resistance is due to a lack of triallate activation (see page 43), treatment with triallate sulfoxide should cause similar

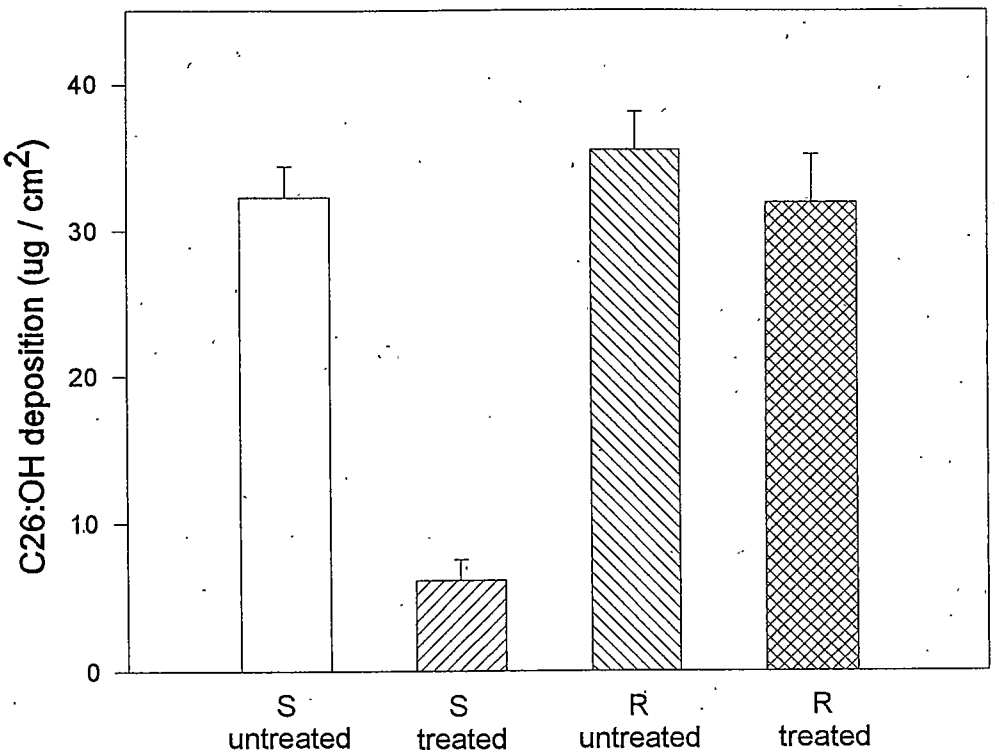

Figure 17. Deposition of new epicuticular wax in R and S wild oats five days after postemergence treatment with 0.1 kg/ha triallate. Vertical error bars represent standard errors of the means.

inhibition between R and S wild oats. To gain better insights into the role triallate sulfoxide plays in this inhibition, I investigated the biosynthesis of wax and wax precursors.

Analysis of Free Fatty Acids.

Due to the dramatic inhibition of epicuticular wax deposition in S seedlings after treatment with triallate, studies were conducted to distinguish between triallate inhibition of short chain fatty acid synthesis, which are the precursors for wax biosynthesis, and the inhibition of elongation reactions. Treatment of etiolated S shoots with 10 ppm triallate did not reduce amounts of C12, C16, C18, or C18:1 fatty acids (Table 4). However, C20, C22, and C26OH constituent levels were significantly reduced. In contrast, none of the fatty acid constituents were affected in R shoots by treatment with 10 or 100 ppm triallate. Reduction of the C26OH wax components agrees with the results of the wax deposition experiment (Figure 17) and supports the findings of Bolton and Harwood (1976), Abulnaja and Harwood (1991a), and Abulnaja and Harwod (1991b), who noted epicuticular wax formation was inhibited by thiocarbamates in susceptible species. Reduction of the C20 and C22 components by triallate in S seedlings has not been previously reported in wild oats; however, biosynthesis of these constituents is thought to rely on the action of fatty acid elongases and a similar inhibition has been reported for other species (Bolton and Harwood, 1976; Harwood, 1992; Harwood, 1994; Kolattukudy and Brown, 1974). The lack of an effect on lipids in R seedlings does not provide insight into the mechanism of resistance, but does support the idea that the herbicide is not reaching or affecting its putative target site. Because previous evidence indicated that triallate resistance in wild oats was due to a decrease in the rate of triallate sulfoxidation (see Chapter 2), I compared the effect of triallate sulfoxide on

TRIALLATE TREATMENT RATE

Lipid	10 ppm			100 ppm		
	S	R	p value	S	R	p value
C_{12}	99	108	0.50	89	104	0.34
C_{16}	88	111	0.17	66	100	0.02
C_{18}	84	97	0.22	69	83	0.27
$C_{18:1}$	104	88	0.18	84	97	0.35
C_{20}	55	106	0.02	40	90	0.0001
C_{22}	28	94	0.001	n.d.	101	0.0000001
$C_{26}OH$	28	96	0.0002	n.d.	87	0.0000003

Table 4. P values were determined by unpaired student's t tests between R and S wild oats 48 hours after treatment for each lipid component as affected by 10 and 100 ppm triallate treatments. n.d.=none detected.

biosynthesis of short- and medium-chain fatty acids in R and S wild oats (Table 5). Treatment of S seedlings with 100 ppm triallate sulfoxide reduced C20, C22, and C26OH amounts to levels very similar to those observed after triallate treatment. However, these fatty acid constituents were also reduced in R seedlings treated with triallate sulfoxide, in contrast to the lack of effect by triallate. These data again support the hypothesis that resistance is conferred by markedly reduced rates of triallate sulfoxidation.

Because of the experimental protocol used here, the data in Tables 4 and

Lipid	TRIALLATE SULFOXIDE TREATMENT RATE					
	100 ppm			500 ppm		
	S	R	p value	S	R	p value
C_{12}	88	94	0.54	81	96	0.23
C_{16}	92	99	0.56	74	98	0.11
C_{18}	86	97	0.40	77	93	0.18
$C_{18:1}$	97	104	0.66	101	103	0.82
C_{20}	48	64	0.14	49	52	0.82
C_{22}	5	22	0.02	n.d.	12	0.26
$C_{26}OH$	n.d.	4	0.34	n.d.	5	0.14

Table 5: P values were determined by unpaired student's t tests between R and S wild oats for each lipid component 48 hours after treatment as affected by 100 and 500 ppm triallate sulfoxide treatments. n.d.=none detected

5 do not differentiate between formation of new fatty acids and those present before treatment. To differentiate between these possibilities, I am currently investigating the effect of triallate and triallate sulfoxide on the incorporation of ^{14}C malonate on the biosynthesis of C_{12} to C_{18} free fatty acids. These data will provide further insights into the mechanism of inhibition of wax biosynthesis by triallate in susceptible wild oats, and will also address the specific inhibition exhibited by triallate sulfoxide.

LITERATURE CITED

Abulnaja, K.O., and Harwood, J.L. 1991a. Thiocarbamate herbicides inhibit fatty acid elongation in a variety of monocotyledons. Phytochem. 30:1445-1447.

Abulnaja, K.O., and Harwood, J.L. 1991b. Interaction of thiocarbamate herbicides with fatty acid synthesis in germinating peas and their microsomal fractions. Phytochem. 30:2883-2887.

Ashton, F.M., and Crafts, A.S. 1981. Bipyridyliums. pp. 164-179 *In* Ashton and Crafts, eds., Mode of action of herbicides. Wiley and Sons, New York.

Babbs, C.F., Pham, J.A., and Coolbaugh, R.C. 1989. Lethal hydroxyl radical production in paraquat-treated plants. Plant Physiol. 90:1267-1270.

Barber, J., and Andersson, B. 1992. Too much of a good thing: light can be bad for photosynthesis. TIBS 17:61-66.

Barrett, P.B., and Harwood, J.L. 1993. The basis of thiocarbamate action on surface lipid synthesis in plants. Brighton Crop Protec. Conf. 12:183-187.

Bishop, T., Powles, S.B., and Cornic, G. 1987. Mechanism of paraquat resistance in *Hordeum glaucum*. II. Paraquat uptake and translocation. Aust. J. Plant Physiol. 14:539-547.

Blackshaw, R.F., O'Donovan, J.T., Sharma, M.P., Harker, K.N., and Maurice, D. 199_. Response of triallate-resistant wild oats (*Avena fatua* L.) to alternative herbicides. Weed Tech. (in press).

Bolton, P., and Harwood, J.L. 1976. Effect of thiocarbamate herbicides on fatty acid synthesis by potato. Phytochemistry 15:1507-1509.

Brown H.M. 1991. Mode of action, crop selectivity, and soil relations of the sulfonylurea herbicides. Pestic. Sci. 29:263-281.

Burnet, M.W.M., Loveys, B.R., Holtum, J.A.M., and Powles, S.B. 1993. A mechanism of chlorotoluron resistance in *Lolium rigidum*. Planta 190:182-198.

Burnet, M.W.M., Loveys, B.R., Holtum, J.A.M., and Powles, S.B. 1993a. Increased detoxification is a mechanism of simazine resistance in *Lolium rigidum*. Pestic. Biochem. Physiol. 46:207-218.

Carringer, R.D., Rieck, C.E., and Bush, L.P. 1978. Metabolism of EPTC in corn (*Zea mays*). Weed Sci. 26:157-160.

Casida, J.E., Kimmel, E.C., Ohkawa, H., and Okhawa, R. 1975. Sulfoxidation of thiocarbamate herbicides and metabolism of thiocarbamate sulfoxides in living mice and liver enzyme systems. Pestic. Biochem. Physiol. 5:1-11.

Christopher, J.T., Powles, S.B., and Holtum, J.A.M. 1992. Resistance to acetolactate synthase-inhibiting herbicides in annual ryegrass (*Lolium rigidum*) involves at least two mechanisms. Plant Physiol. 100:1909-1913.

Christopher, J.T., Powles, S.B., Liljegren, D.R., and Holtum, J.A.M. 1991. Cross resistance to herbicides in annual ryegrass (*Lolium rigidum*). II. Chlorsulfuron resistance involves a wheat-like detoxification system. Plant Physiol. 95:1036-1043.

Clipsham, I.D. 1985. Effects of surfactants on uptake and movement of ^{14}C difenzoquat in *Avena fatua*, wheat and barley. Asp. Appl. Biol. 9:141-149.

Cotterman, J.C., and Saari, L.L. 1992. Rapid metabolic inactivation is the basis for cross-resistance to chlorsulfuron in diclofop-methyl-resistant rigid ryegrass (*Lolium rigidum*) SR4/84. Pestic. Biochem. Physiol. 43:182-192.

Coupland, D. 1994. Resistance to the auxin analog herbicides. pp. 171-214 *In* Powles, S.B. and Holtum, J.A.M., eds., Herbicide Resistance in Plants-Biology and Biochemistry. Lewis Publishers, London.

Davis, D., and Linscott, D.L. 1986. Tolerance of birdsfoot trefoil (*Lotus cornicalatus*) to 2,4-D. Weed Sci. 34:373-376.

Dean, J.V., Gronwald, J.W., and Anderson, M.P. 1991. Glutathione S-transferase activity in nontreated and CGA-154281-treated maize shoots. Z. Naturforsch. 46c:850-855.

Devine, M.D., Marles, M.A.S., and Hall, L.M. 1991. Inhibition of acetolactate synthase in susceptible and resistant biotypes of *Stellaria media*. Pestic. Sci. 31:273-280.

Devine, M.D., Renault, S., and Wang, X. 1993. Alternative mechanisms of resistance to acetyl-CoA carboxylase inhibitors in grass weeds. pp. 541-548 *In* Proc. British Crop Protection Conference- Weeds. Farnham, U.K.

Devine, M.D., and Shimabukuro, R.H. 1994. Resistance to acetyl-coenzyme A carboxylase inhibiting herbicides. pp. 141-169 *In* Powles, S.B. and Holtum, J.A.M., eds., Herbicide Resistance in Plants- Biology and Biochemistry. Lewis Publishers, London.

Ezra, G., Gressel, J., and Flowers, H.M. 1983. Effects of the herbicide EPTC and the protectant DDCA on incorporation and distribution of [2-^{14}C] acetate into major lipid fractions of maize cell suspension cultures. Pestic. Biochem. Physiol. 19:225-234.

Faulkner, J.S. 1976. A paraquat resistant variety of *Lolium perenne* under field conditions. pp 485-490 *In* Brighton Crop Protection Conference- Weeds. Farnham, U.K.

Fonne-Pfister, R., Gaudin, J., Kreuz, K., Ramsteiner, K., and Ebert, E. 1990. Hydroxylation of primisulfuron by an inducible cytochrome P450-dependent monooxygenase system from maize. Pestic. Biochem. Physiol. 37:165-173.

Frear, D.S., Swanson, H.R., and Thalacker, F.W. 1991. Induced microsomal oxidation of diclofop, triasulfuron, chlorsulfuron, and linuron in wheat. Pestic. Biochem. Physiol. 41:274-287.

Fridovich, I. 1983. Superoxide radical: an endogenous toxicant. Annu. Rev. pharmacol. Toxicol. 23:239-257.

Friesen, L.F. 1992. Herbicide resistance summary. pp. 408-413 *In* Research report, Expert Committee on Weeds. Agriculture Canada.

Fuerst, E.P., Nakatani, H.Y., Dodge, A.D., Penner, D., and Arntzen, C.J. 1985. Paraquat resistance in *Conyza*. Plant Physiol. 77:984-989.

Fuerst, E.P., and Norman, M.A. 1991. Interactions of herbicides with photosynthetic electron transport. Weed Sci. 39:458-464

Gentner, W.A. 1966. The influence of EPTC on external foliage wax deposition. Weeds 14:27-31.

Golbeck, J.H., and Cornelius, J.M. 1986. Photosystem I charge separation in the absence of centers A and B. I. Optical characterization of centre A_2 and evidence for its association with a 64-kDa protein. Biochim. Biophys. Acta 849:16-24.

Gonneau, M., Pasquette, B., Cabanne, F., and Scalla, R. 1988. Metabolism of chlortoluron in tolerant species: possible role of cytochrome P-450 monooxygenases. Weed Res. 28:19-25.

Gronwald, J. W. 1994. Resistance to photosystem II inhibiting herbicides. *In* Powles, S.B. and Holtum, J.A.M., eds., Herbicide Resistance in Plants- Biology and Biochemistry. Lewis Publishers, London.

Gronwald, J.W., Andersen, R.N., and Yee, C. 1989. Atrazine resistance in velvetleaf (*Abutilon theophrasti*) due to enhanced atrazine detoxification. Pestic. Biochem. Physiol. 34:149-163.

Hackett, A.G., Kotyk, J.J., Fujiwara, H., and Logusch, E.W. 1993. Metabolism of triallate in Sprague-Dawley rts. 3. In vitro metabolic pathways. J. Aric. Food Chem. 41:141:147.

Hall, L.M., Holtum,J.A.M., and Powles, S.B. 1994. Mechanisms responsible for cross resistance and multiple resistance. pp. 243-261 *In* Powles, S.B. and Holtum, J.A.M., eds., Herbicide Resistance in Plants- Biology and Biochemistry. Lewis Publishers, London.

Halling, B.P., and Behrens, R. 1983. Effects of difenzoquat on photoreactions and respiration in wheat (*Triticum aestivum*) and wild oat (*Avena fatua*). Weed Sci. 31:693-699.

Hart, J.J., DiTomaso, J.M., Linscott, D.L., and Kochian, L.V. 1992. Characterization of the transport and cellular compartmentalization of paraquat in roots of intact maize seedlings. Pestic. Biochem. Physiol. 43:212-222.

Harwood, J.L., Ridley, S.M., and Walker, K.A. 1989. Herbicides inhibiting lipid synthesis. pp. 73-96 *In* Harwood, J.L., ed., Herbicides and Plant Metabolism. Cambridge University Press, New York.

Harwood, J.L., and Stumpf, P.K. 1971. Fat metabolism in higher plants: control of fatty acid synthesis in germinating seeds. Arch. Biochem. Biophys. 142:281-291.

Häusler, R.E., Holtum, J.A.M., and Powles, S.B. 1991. Cross resistance to herbicides in annual ryegrass (*Lolium rigidum*). IV. Correlation between membrane effects and resistance to graminicides. Plant Physiol. 97:1034-1043.

Hess, F.D. 1987. Herbicide effects on the cell cycle of meristematic plant cells. Rev. Weed Sci. 3:183-203.

Hubbell, J.P., and Casida, J.E. 1977. Metabolic fate of the *N,N*-dialkylcarbamoyl moiety of thiocarbamate herbicides in rats and corn. J. Agric. Food Chem. 25:404-413.

Jablonkai, I., and Hatzios, K.K. 1994. Microsomal oxidation of the herbicides EPTC and acetochlor and of the safener MG-191 in maize. Pestic. Biochem. Physiol. 48:98-109.

Jacobson, A., and Shimabukuro, R.H. 1984. Metabolism of diclofop-methyl in root-treated wheat and oat seedlings. J. Agric. Food Chem. 32:742-746.

Jana., S., and Naylor, J.M. 1976. Genetic adaptation for seed dormancy in *Avena fatua*. Can. J. Bot. 54:306-312.

Jana, S., and Naylor, J.M. 1982. Adaptation for herbicide tolerance in populations of *Avena fatua*. Can. J. Box. 60:1611-1617.

Kappus, H. 1986. Overview of enzyme systems involved in bio-reduction of drugs and in redox cycling. Biochem. Pharmacol. 35:1-6.

Kern, A.J., Colliver, C.T., Cranston, H.J., and Dyer, W.E. 1994. Triallate resistant wild oats are cross-resistant to difenzoquat. Proc. West. Soc. Weed Sci. 47:105.

Kern, A.J., and Dyer, W.E. 1995. Triallate and difenzoquat metabolism studies in resistant wild oats (*Avena fatua* L.). Abs. Weed Sci. Soc. Amer. 35:66.

Kolattukudy, P.E., and Brown, L. 1974. Inhibition of cuticular lipid biosynthesis in *Pisum sativum* by thiocarbamates. Plant Physiol. 53:903-906.

Lay, M.M., and Casida, J.E. 1976. Dichloroacetamide antidotes enhance thiocarbamate sulfoxide detoxification by elevating corn root glutathione content and glutathione *S*-transferase activity. Pestic. Biochem. Physiol. 6:442-456.

Lay, M.M., Hubbell, J.P., and Casida, J.E. 1975. Dichloroacetamide antidotes for thiocarbamate herbicides: mode of action. Science 189:287-288.

Leavitt, J.R.C., Duncan, D.N., Penner, D., and Meggitt, W.F. 1978. Inhibition of epicuticular wax deposition on cabbage by ethofumesate. Plant Physiol. 61:1034-1036.

Leavitt, J.R.C., and Penner, D. 1979. In vitro conjugation of glutathione and other thiols with acetanilide herbicides and EPTC sulfoxide and the action of the herbicide antidote R-25788. J. Agric. Food Chem. 27:533-536.

LeBaron, H.M. 1991. Distribution and seriousness of herbicide-resistant weed infestations worldwide. pp. 27-43 *In* Caseley, J.C., Cussans, G.W., and Atkin, R.K., eds., Herbicide Resistance in Weeds and Crops. Butterworth-Heinemann, Oxford.

Leckie, D., and Snape, J.W. 1992. The location and effects of genes modifying the response of wheat to the herbicide difenzoquat. J. Agricultural Sci. 118:9-15.

Malchow, W.E. 1995. Wild oat resistance to triallate in Montana. M.S. Thesis. Montana State University.

Malchow, W.E., Maxwell, B.D., Fay, P.K., and Dyer, W.E. 1993. Frequency of triallate resistance in Montana. Proc. West. Soc. Weed Sci. 46:75.

Mallory-Smith, C.A., Thill, D.C., and Dial, M.J. 1990. Identification of sulfonylurea herbicide-resistant prickly lettuce (*Lactuca serriola*). Weed Tech. 4:163-168.

Marshall, G., Kirkwood, R.C., and Martin, D.J. 1987. Studies on the mode of action of asulam, aminotriazole and glyphosate in *Equisetum arvense* L. (Field Horsetail). II. The metabolism of [^{14}C]asulam, [^{14}C]aminotriazole and [^{14}C]glyphosate. Pestic. Sci. 18:65-77.

Matthews, J.M., Holtum, J.A.M., Liljegren, D.R., Furness, B., and Powles, S.B. 1990. Cross-resistance to herbicides in annual ryegrass (*Lolium rigidum*). I. Properties of the herbicide target enzymes acetyl coenzyme-A carboxylase and acetolactate synthase. Plant Physiol. 94:1180-1186.

Maxwell, B.D., and Mortimer, A.M. 1994. Selection for herbicide resistance. pp 1-25 *In* Powles, S.B. and Holtum, J.A.M., eds., Herbicide Resistance in Plants- Biology and Biochemistry. Lewis Publishers, London.

Mets, L., and Thiel, A. 1989. Biochemistry and genetic control of the photosystem II herbicide target site. pp 1-24 *In* Böger, P. and Sandmann, G., eds., Target Sites of Herbicide Action. CRC Press, Boca Raton, Fl.

Meyer, A.M., and Muller, F. 1989. Triasulfuron and its selective behaviour in wheat and *Lolium perenne*. pp. 441-448 *In* Brighton Crop Protection Conference-Weeds. Farnham, U.K

Morden, C.W., and Golden, S.S. 1989. *psb*A genes indicate common ancestry of prochlorophytes and chloroplasts. Nature 337:382-385.

Morejohn, L.C., Bureau, T.E., Mole-Bajer, J., Bajer, A.S., and Fosket, D.E. 1987. Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro. Planta 172:252-264.

Morrison, I.N., Todd, B.G., and Nawolsky, K.M. 1989. Confirmation of trifluralin-resistant green foxtail (*Setaria viridis*) in Manitoba. Weed Tech. 3:544-551.

Mudge, L.C., Gossett, B.J., and Myrphy, T.R. 1984. Resistance of goosegrass (*Eleusine indica*) to dinitroaniline herbicides. Weed Sci. 32:591-594.

Murray, B.G., Morrison, I.N., and Brûlé-Batel. 199_. Inheritance of acetyl-CoA carboxylase inhibitor resistance in wild oats (*Avena fatua*). Weed Sci. (in press).

O'Donovan, J.T. 1995. Unpublished data; personal communication.

O'Donovan, J.T., Sharma, M.P., Harker, K.N., Maurice, D., Baig, M.N., and Blackshaw, R.E. 1994. Wild oats (*Avena fatua*) populations resistant to triallate are also resistant to difenzoquat. Weed Sci. 42:195-199.

Omer, C.A., Lenstra, R., Litle, P.J., Dean, C., Tepperman, J.M., Leto, K.J., Romesser, J.A., and O'Keefe, D.P. 1990. Genes for two herbicide-inducible cytochrome P450s from *Streptomyces griseolus*. J. Bacteriol. 172:3335-3345.

Pfister, K., and Arntzen, C.J. 1979. The mode of action of photosystem II-specific inhibitors in herbicide-resistant weed biotypes. Z. Naturforsch. 34c:996-1009.

Powles, S.B., and Cornic, G. 1987. Mechanism of paraquat resistance in *Hordeum glaucum*. I. Studies with isolated organelles and enzymes. Aust. J. Plant Physiol. 14:81-89.

Preston, C., Holtum, J.A.M., and Powles, S.B. 1992. Do polyamines contribute to paraquat resistance in *Hordeum glaucum*? *In* Murata, N., ed., Research in Photosynthesis, Vol. III. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Preston, C. 1994. Resistance to photosystem I distupting herbicides. pp. 61-82 *In* Powles, S.B. and Holtum, J.A.M., eds., Herbicide Resistance in Plants-Biology and Biochemistry. Lewis Publishers, London.

Rajasekharan, R., and Sastry, P.S. 1989. Effect of thiocarbamate, urea, and uracil herbicides on lipid metabolism in groundnut (*Arachis hypogaea)* leaves. Biochem. Cell Biol. 68:567-573.

Ritter, R.L. 1986. Triazine resistant velvetleaf and giant foxtail control in no-tillage corn. Proc. Northeastern Weed Sci. Soc. 40:50-52.

Ryan, G.F. 1970. Resistance of Common Groundsel to Simazine and Atrazine. Weed Sci. 18:614-616.

Saari, L.L., Cotterman, J.C., Smith, W.F., and Primiani, M.M. 1992. Sulfonylurea herbicide resistance in common chickweed, perennial ryegrass and russian thistle. Pestic. Biochem. Physiol. 42:110-118.

Saari, L.L., Cotterman, J.C., and Thill, D.C. 1994. Resistance to acetolactate synthase inhibiting herbicides. pp. 83-139 *In* Powles, S.B. and Holtum, J.A.M., eds., Herbicide Resistance in Plants- Biology and Biochemistry. Lewis Publishers, London.

Schmitt, R., and Sandermann, H. 1982. Specific localisation of β-D-glucoside conjugates of 2,4-dichlorophenoxyacetic acid in soybean vacuoles. Z. Naturforsch 37:772-777.

Schuphan, I., and Casida, J.E. 1979a. *S*-chloroallyl thiocarbamate herbicides: chemical and biological formation and rearrangement of diallate and triallate sulfoxides. J. Agric. Food Chem. 27:1060-1066.

Schuphan, I., and Casida, J.E. 1979b. [2,3] sigmatropic rearrangement of *S*-(3-chloroallyl) thiocarbamate sulfoxides followed by a 1,2-elimination reaction yielding unsaturated aldehydes and acid chlorides. Tetrahedron Lett. 10:841-844.

Schuphan, I., Rosen, J.D., and Casida, J.E. 1979. Novel activation mechanism for the promutagenic herbicide diallate. Science 205:1013-1015.

Seefeldt, S.S., Jensen, J.E., and Fuerst, E.P. 1995. Log-logistic analysis of herbicide dose-response relationships. Weed Tech. 9:218-227.

Shaner, D.L. 1983. Mode of action of Avenge (difenzoquat). Can. Plant Proc. 12:49-58.

Shaner, D., Sinha, A., and Braddock, R. 1992. Designing strategies to delay development of resistance to herbicides. Proc. of the First Int. Weed Control Conf. 1:236-239.

Sharma, M.P., Vanden Born, W.H., Friesen, H.A., and McBeath, D.K. 1976. Penetration, translocation, and metabolism of ^{14}C-difenzoquat in wild oat and barley. Weed Sci. 24:379-384.

Smeda, R.J., and Vaughn, K.C. 1994. Resistance to dinitroaniline herbicides. pp. 215-228 *In* Powles, S.B. and Holtum, J.A.M., eds., Herbicide Resistance in Plants- Biology and Biochemistry. Lewis Publishers, .

Smeda, R.J., Vaughn, K.C., and Morrison, I.N. 1992. A novel pattern of herbicide cross-resistance in a trifluralin-resistant biotype of green foxtail (*Setaria viridis* (L.) Beauv.). Pestic. Biochem. Physiol. 42:227-241.

Snape, J.W., Angus, W.J., Parker, B.B., and Leckie, D. 1987. The chromosomal locations in wheat of genes conferring differential response to the wild oat herbicide difenzoquat. J. Agricultural Sci. 108:543-548.

Still, G.G., Davis, D.G., and Zander, G.L. 1970. Plant epicuticular lipids: alteration by herbicidal carbamates. Plant Physiol. 46:307-314.

Streibig, J.C., and Kudsk, P. Herbicide Assays. CRC Press, Boca Raton, Fl.

Suttle, J.C., and Hulstrand, J.F. 1984. Growth retarding effects of difenzoquat. Plant Physiol. 75:88-93.

Sweetser, P.B., Schow, G.S., and Hutchison, J.M. 1982. Metabolism of chlorsulfuron by plants: biological basis for selectivity of a new herbicide for cereals. Pestic. Biochem. Physiol. 17:18-23.

Tanaka, Y., Chisaka, H., and Saka, H. 1986. Movement of paraquat in resistant and susceptible biotypes of *Erigeron philadelphicus* and *E. canadensis*. Physiol. Plant. 66:605-608.

Thai, K.M., Jana, S., and Fowke, L.C. Cell membrane permeability and ultrastructural effects of difenzoquat on wild oats (*Avena fatua*). Weed Sci. 37:98-106.

Thai, K.M., Jana, S., and Naylor, J.M. 1985. Variability for response to herbicides in wild oat (*Avena fatua*) populations. Weed Sci. 33:829-835.

Thurston, J.M., and Phillipson, A. 1976. Distribution of wild oats in world agriculture. pp. 19-64 *In* Jones, D.P., ed., Wild Oats in World Agriculture. Agricultural Research Council. London.

Tietjen, J.G., Kluth, J.F., Andree, R., Haug, M., Lindig, M., Müller, K.H., Wroblowsky, H.J., and Trebst, A. 1991. The herbicide binding niche of photosystem II-a model. Pestic. Sci. 31:65-72.

Timmerman, K.P. 1989. Molecular characterization of corn glutathione S-transferase isozymes involved in herbicide detoxication. Physiol. Plant. 77:465-471.

Tischer, W., and Strotmann, H. 1977. Relationship between inhibitor binding by chloroplasts and inhibition of photosynthetic electron transport. Biochim. Biophys. Acta 460:113-125.

Trebst, A. 1991. The molecular basis of resistance of photosystem II herbicides. pp. 145-164 *In* Caseley, J.C., Cussans, G.W., and Atkin, R.K., eds., Herbicide Resistance in Weeds and Crops. Butterworth-Heinemann, Oxford.

Trebst, A. 1987. The Three-dimensional structure of the herbicide binding niche on the reaction center polypeptides of photosystem II. Z. Naturforsch. 42c:742-750.

Vaughn, K.C., Vaughan, M.A., and Camilleri, P. 1989. Lack of cross-tolerance of paraquat-resistant hairy fleabane (*Conyza bonariensis*) to other toxic oxygen generators indicates enzymatic protection is not the resistance mechanism. Weed Sci. 37:5-11.

Vermass, W.F.J., and Arntzen, C.J. 1983. Synthetic quinones influencing herbicide binding and photosystem II electron transport: the effects of triazine-resistance on quinone binding properties in thylakoid membranes. Biochim. Biophys. Acta 725:483-491.

Vermass, W. F.J., Renger, G., and Arntzen, C.J. 1984. Herbicide/quinone binding interactions in photosystem II. Z. Naturforsch. 39c:368-373.

Wilkinson, R.E. 1974. Sicklepod surface wax response to photoperiod and *S*-(2,3-dichloroallyl)diisopropylthiocarbamate (diallate). Plant Physiol. 53:269-275.

Wilkinson, R.E. 1983(a). Gibberellin precursor biosynthesis by EPTC and reversal by R-25788. Pestic. Biochem. Physiol. 19:321-329.

Wilkinson, R.E. 1983(b). Metabolism of ^{14}C vernolate in soybeans. Pestic. Biochem. Physiol. 20:347-353.

Wilkinson, R.E. 1986. Diallate inhibition of gibberellin biosynthesis in sorghum coleoptiles. Pestic. Biochem. Physiol. 25:93-97.

Wilkinson, R.E., and Hardcastle, W.S. 1969. EPTC effects on total leaflet fatty acids and hydrocarbons. Weed Sci. 4:125-128.

Zimmerlin, A., and Durst, F. 1990. Xenobiotic metabolism in plants: arylhydroxylation of diclofop by a cyrochrome-P-450 enzyme from wheat. Phytochemistry 29:88-96.

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