

BASIS FOR THE SAFENING OF THE COTTON FROM HERBICIDE
CLOMAZONE BY THE ORGANOPHOSPHATE INSECTICIDE PHORATE AND
STUDIES OF THE CLOMAZONE MODE OF ACTION

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy
in the College of Agriculture at the University of Kentucky

By

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Lexington, Kentucky

Director: Dr. Michael Barrett, Professor of Agronomy

Lexington, Kentucky

2002

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metabolism in roots of intact cotton plants. HPLC analysis of polar metabolites extracted from cotton shoots showed no clear reduction in any single polar metabolite. The majority of the polar metabolites were conjugates of clomazone metabolites.

Treatment of 7-d-old cotton seedlings with 5-OH clomazone (a clomazone metabolite) decreased chlorophyll and carotenoid contents similarly as clomazone treatment. Phorate did not safen 7-d-old cotton seedlings from 100 nM 5-OH clomazone.

Clomazone, 5-OH clomazone, and 5-keto clomazone did not inhibit formation of products from isopentenyl pyrophosphate, an intermediate in the chloroplastic isoprenoid pathway. However, when pyruvate, a substrate for the 1st committed step of the pathway, was used as a precursor, 5-keto clomazone inhibited the formation of a non-polar product that cochromatographed with lutein. Neither clomazone nor 5-OH clomazone treatment produced this inhibition. Additionally, one polar product formed with pyruvate feeding was reduced with 5-keto clomazone. In an in-vitro assay, 5-keto clomazone, but not clomazone or 5-OH clomazone, inhibited DOXP synthase, the enzyme catalyzing the first committed step of the pathway.

KEYWORDS: Clomazone, 5-keto clomazone, Phorate, Safening, Mode of Action

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ACKNOWLEDGEMENT

I wish to thank my advisor, Dr. Michael Barrett for his friendship, guidance, and support throughout my Doctorate program. I sincerely appreciate his many efforts on my behalf. I would also like to thank the other members of my committee, Drs. William W. Witt, J. D. Green, Lowell Bush, and George Wagner who provided invaluable insight and criticism.

I would like to recognize Dr. Joe Chappell for his guidance on my project . Additionally, I wish to thank Charles H. Slack for his teaching me weed management in the summer training in the field. I also wish to thank my fellow graduate students Ann Freytag, Jennifer Lambert, Chad Brommer, Mike Marshall, Andy Lee, Michael Obermeier, Shea Murdock, Jay Ferrell, Joe Settles, and Justin Ewing who have assisted, advised, and supported me during my education at the University of Kentucky.

Lastly and most importantly, I would like to thank my husband Burak Ferhatoglu for his continuing support, understanding, and encouragement throughout my program. Finally, I would like to thank our daughter Asli who always brought a smile to the end of every day.

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Chapter Two

Basis for the Safening of the Cotton from the Herbicide Clomazone by the Organophosphate Insecticide Phorate

INTRODUCTION

There are numerous examples where the simultaneous or sequential application of insecticides and herbicides to a crop alters the response of plants to the herbicide. For example, bentazon (1H-2,1,3-benzothiadiazin-4(3H)-one, 3-(1-methylethyl)-one 2,2-dioxide) foliar applied with the organophosphate (OP) insecticides malathion ([dimethoxyphosphinothioyl]thio] butanedioic acid, diethyl ester), parathion (phosphorothioic acid, O,O-diethyl O-(4-nitrophenyl) ester), or diazinon (phosphorothioic acid, O,O-diethyl O-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] ester), but not the carbamate insecticides carbaryl (1-naphthalenol, methylcarbamate) or carbofuron (7-benzafuranol, 2,3-dihydro-2,2-dimethyl, methylcarbamate), caused injury to bentazon tolerant soybean (*Glycine max*) and navybean (*Phaseolus vulgaris*) (Chample & Shaner, 1982). Combinations of carbaryl, chlorpyrifos (phosphorothioic acid, O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) ester), malathion (diethyl (dimethoxythiophosphorylthio) succinate), or methomyl (s-methyl N-(methylcarbamoyloxy)thioacetimidate) with thifensulfuron (methyl 3-[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]-2-thiophenecarboxylate) also caused soybean injury (Ahrens, 1990). A recent example of greater crop injury from herbicide and insecticide combinations is the increased injury to corn (*Zea mays*) from nicosulfuron (2-((((4,6-dimethoxy-2-pyrimidinyl)amino)carbonyl)aminosulfonyl)-N,N-dimethyl-3-pyridinecarboxamide) or primisulfuron (1-(2-methoxy-carbonylphenylsulfonyl)-3-[4,6-bis(ethoxycarbonyl)-ethyl-O,O-dimethylphosphorodithioate) when used after soil-applied insecticides (Biediger et al., 1992; Kapusta and Krausz,

1992; Morton et al., 1993; Morton et al., 1991; Porpiglia et al., 1990; Rahman and James, 1993). OP insecticides cause nicosulfuron or primisulfuron injury to corn while none of the carbamate or pyrethroid insecticides tested increased maize injury from these herbicides (Biediger et al., 1992; Kapusta & Krausz, 1992; Porpiglia et al., 1990). Of the OP insecticides, terbufos (S-[[[(1,1-dimethylethyl)thio]methyl]O,O-diethyl phosphorodithioate) was most likely to cause herbicide injury on corn (Biediger et al., 1992; Kapusta & Krausz, 1992; Porpiglia et al., 1990). Other OP insecticides such as phorate (phosphorodithioic acid, O,O-diethyl S-[(ethylthio)methyl]ester), fonofos (phosphonodithioic acid, ethyl-, O-ethyl S- phenyl ester), malathion, chlorpyrifos, disulfoton (phosphorodithioic acid, O,O-diethyl S-[2-(ethylthio)ethyl]ester), and isazophos (O-5-chloro-1-isopropyl-1H-1,2,4-triazol-3-yl O,O-diethyl phosphorothioate) also have the potential to promote nicosulfuron or primisulfuron injury to corn, especially if they are applied in-furrow or as a mixture with the foliar applied herbicides (Biediger et al., 1992; Kapusta & Krausz, 1992; Porpiglia et al., 1990). The OP insecticides diazinon, parathion, and methomyl are not likely to result in corn injury when applied prior to or with nicosulfuron or primisulfuron (Biediger et al., 1992; Porpiglia et al., 1990).

Tolerance of crops to herbicides is most commonly based on rapid herbicide metabolism (Chang et al., 1971) and certain insecticides can decrease the rate of herbicide metabolism (Chang et al., 1971; Diehl, et al., 1995; Frazier et al., 1993, Swanson & Swanson, 1964). Rice (*Oryza sativa*) tolerance of propanil (3',4'-dichloropropionanilide) is due to deesterification of the herbicide by aryl acylamidases (Frear & Still, 1968; Matsunaka, 1968). OP insecticides (Frear & Still, 1968) and their oxidized metabolites (Matsunaka, 1968), as well as carbamates (Yih et al., 1968), inhibit this reaction (Frear & Still, 1968; Matsunaka, 1968; Yih et al., 1968) resulting in decreased propanil metabolism (Yih et al., 1968) and increased rice injury (Matsunaka, 1968). Certain carbamate insecticides also inhibit the demethylation of monuron (3-

(p-chlorophenyl)-1,1-dimethylurea mono(trichloroacetate)) in cotton slowing herbicide detoxification and prolonging photosynthetic inhibition by the herbicide (Swanson & Swanson, 1964).

Clomazone (2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone) was registered for use in cotton (*Gossypium hirsutum*.) in 1993. It may be applied as a preemergent banded or broadcast treatment in conventional tillage or conservation tillage systems in cotton. However, for adequate crop safety, phorate or disulfoton must be applied in-furrow with the seed when clomazone is used at planting time (Command Label., Hayes et al., 1993, Harrison et al., 1992). Use of other insecticides, such as aldicarb [(2-methyl-2-methylthio) propionaldehyde O-(methylcarbamoyl) oxime]] in conjunction with clomazone can reduce the safening effect of disulfoton or phorate (Guy et al., 1994). Recently, it was shown that protection of cotton from clomazone injury by disulfoton or phorate was not dependent on reduced absorption or translocation of clomazone (Culpepper et al., 2001). Despite the use of the OP insecticides phorate or disulfoton as safeners for clomazone use in cotton for several years, the mechanism of safening has not been established.

The initial metabolites of clomazone in soybean are all hydroxylated derivatives (El Naggar et al., 1992) which are prime candidates to be the products of a cytochrome P450 monooxygenase(s) (P450s) reaction(s). Although the metabolic pathway of clomazone in cotton has not been published, the clomazone metabolic pathway in soybeans suggests that a P450(s) could metabolize the herbicide in cotton.

OP insecticides are known to inhibit P450 mediated detoxification. Malathion inhibited the in vitro metabolism of primisulfuron in maize microsomes (Kreuz and Pfister, 1992). The inhibition was proposed to occur through a mechanism-based or suicide substrate P450 inactivation (Kreuz and Pfister, 1992). Later studies found malathion was also an effective inhibitor of bentazon, imazethapyr ((±)[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-ethyl-3-pyridinecarboxylic acid) and nicosulfuron metabolism

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Treatment of 7-d-old cotton seedlings with 5-OH clomazone (a clomazone metabolite) decreased chlorophyll and carotenoid contents similarly as clomazone treatment. Phorate did not safen 7-d-old cotton seedlings from 100 nM 5-OH clomazone.

Clomazone, 5-OH clomazone, and 5-keto clomazone did not inhibit formation of products from isopentenyl pyrophosphate, an intermediate in the chloroplastic isoprenoid pathway. However, when pyruvate, a substrate for the 1st committed step of the pathway, was used as a precursor, 5-keto clomazone inhibited the formation of a non-polar product that cochromatographed with lutein. Neither clomazone nor 5-OH clomazone treatment produced this inhibition. Additionally, one polar product formed with pyruvate feeding was reduced with 5-keto clomazone. In an in-vitro assay, 5-keto clomazone, but not clomazone or 5-OH clomazone, inhibited DOXP synthase, the enzyme catalyzing the first committed step of the pathway.

KEYWORDS: Clomazone, 5-keto clomazone, Phorate, Safening, Mode of Action

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Chapter One

Literature Review

Clomazone (2-[(2-chlorophenyl) methyl]-4, 4-dimethyl-3-isoxazolidinone) was developed by FMC Corporation for control of many species of annual broad-leaved and grassy weeds (Warfield et al., 1985; Chang et al., 1987).

Clomazone, a selective soil-applied herbicide, is registered for use in pepper (*Capsicum annum*), pumpkin (*Cucurbita pepo*), soybean (*Glycine max*), cotton (*Gossypium hirsutum*), tobacco (*Nicotiana tabacum*), and pea (*Pisum sativum*) (Blackshaw and Esua 1991; Jordan et al., 1993; Langton et al., 1997; Westburg et al., 1989). Clomazone, known as a bleacher herbicide, interferes with chloroplast development (Duke et al., 1985) resulting in a white, yellow or pale green appearance in susceptible seedlings.

Clomazone can be an effective component of cotton weed management programs (Jordan et al., 1993). However, excessive crop injury was noted in initial studies with clomazone applied preplant incorporated or preemergence to cotton (Applewhite et al., 1988). Four-leaf or larger cotton plants tolerated clomazone applied postemergence-directed but the utility of clomazone applied in this manner was limited because emerged weeds were not controlled. In later studies (York and Jordan, 1992; York et al., 1991), antagonism between clomazone and organophosphate insecticides (OPs) was noted on cotton. Cotton tolerated clomazone applied preplant incorporated when the insecticides disulfoton [phosphorodithioic acid, *O,O*-diethyl *S*-[2-(ethylthio)ethyl]ester] or phorate [phosphorodithioic acid, *O,O*-diethyl *S*-[(ethylthio)methyl]ester]

were applied in the seed furrow. Clomazone applied alone or in combination with aldicarb [2-methyl-2-methylthio) propionaldehyde *O*-(methylcarbamoyl) oxime] in the seed furrow caused cotton injury and reduced cotton stands (York et al., 1991). In contrast, clomazone in combination with disulfoton or

phorate injured cotton less and did not reduce stands. Clomazone was registered for use in cotton as a preemergent banded or broadcast treatment in conventional tillage or conservation tillage systems in cotton in 1993. However, for adequate crop safety, phorate or disulfoton must be applied in furrow with the seed when clomazone is used at planting time (Command Label., Hayes et al., 1993, Harrison et al., 1992). Until today, the mechanism for the interaction between clomazone and OPs on cotton has not been elucidated.

Clomazone is absorbed by roots and shoots and is translocated in the xylem and then diffuses within the leaf (Weimer et al., 1991; Weston & Barrett, 1989; Vencill et al., 1990; Goetz et al., 1988). Uptake, translocation, and metabolism of clomazone were examined to explain differential clomazone tolerance between soybean, common cocklebur (*Xanthium strumarium*), sicklepod (*Cassia obtusifolia*), and velvetleaf (*Abutilon theophrasti*). The order of species clomazone tolerance, measured by chlorophyll content following clomazone treatment, was soybean > common cocklebur = sicklepod > velvetleaf. Clomazone absorption through the roots was greater than through cotyledons and hypocotyls in tested species. Soybean, regardless of soil application zone (root or cotyledons and hypocotyls), absorbed more clomazone than the other species. Translocation from roots to shoots varied between 43 and 69% for the four species. Translocation from shoot to roots was 21 to 23% of the absorbed herbicide for all species (Goetz et al., 1988). However, in another study, even though velvetleaf was 162 times more sensitive to clomazone than soybean, both species had similar clomazone uptake and translocation (Weimer et al., 1991). Clomazone did not accumulate in the roots but was rapidly translocated to the shoots in both species. Bell pepper is 40 times more tolerant to clomazone than tomato (*Lycopersicon esculentum*) but differences in clomazone uptake and translocation were not related to selectivity between the species (Weston & Barrett, 1989). However, differential root absorption appeared to play a significant role in the differential

response between clomazone tolerant soybean and *Amaranthus hybridus* and susceptible *A. retroflexus* and *A. lividus* (Vencill et al., 1990). At 96 h after treatment, greater than 80% of the applied ^{14}C -clomazone was absorbed by *A. retroflexus* and *A. lividus*. During the same time period, absorption of ^{14}C -clomazone by soybean and *A. hybridus* was 53 and 34%, respectively. Of the absorbed radioactivity, 61 to 80 percent was translocated to the leaves of the four plant species following application of ^{14}C -clomazone to the roots. Thus, in terms of efficiency in absorbing root-applied clomazone, the four species ranked as follows: *A. lividus* > *A. retroflexus* > soybean > *A. hybridus*. Movement of the absorbed radioactivity from roots to shoots and leaves appeared to be independent of the time after treatment of root-applied ^{14}C -clomazone in all four species.

Generally, the most important herbicide selectivity mechanism in plants (crops or weeds) is differential (rate or route of) metabolism but a differential rate of clomazone metabolism between sensitive and tolerant plants was not observed in studies of clomazone metabolism by sensitive *Amaranthus* species (*Amaranthus hybridus*, *A. retroflexus*, and *A. lividus*) and tolerant soybean (Vencill et al., 1990). Fifty percent of the absorbed ^{14}C -clomazone was metabolized by soybean and *Amaranthus* species as early as 12 h after treatment. (Vencill et al., 1990). In addition, studies with clomazone tolerant bell pepper and clomazone sensitive tomato showed there was little difference in the quantitative or qualitative metabolism of the herbicide by the two species (Weston and Barrett, 1989). Similarly, there was no difference in the rate of clomazone metabolism between cole crops (*Brassica oleracea*) of varying clomazone sensitivity (Scott, 1992). Similarly, four days after emergence, clomazone tolerant soybean metabolized 82% of the absorbed clomazone while the most clomazone sensitive species, velvetleaf, had metabolized only 17% of the absorbed herbicide (Goetz et al., 1988).

Similar clomazone metabolites, more polar than clomazone, were formed in clomazone sensitive velvetleaf and tolerant soybean. Metabolism of

the herbicide is actually more rapid in velvetleaf than in soybean (Weimer et al., 1991). This suggested that a bioactivation of clomazone was occurring within the sensitive plants. Norman et al. (1990b) compared metabolism of clomazone in tolerant soybean cell cultures to that in susceptible cotton cultures and found that, despite the approximately 20 fold differences in clomazone sensitivity between two types of cell lines, there was no difference in the amount of clomazone metabolism between them 48 hours after treatment. Soybean cells did have less clomazone present in them 6 and 34 hours after treatment. However, most significantly, clomazone metabolites isolated from cotton cells, but not those from soybean cells, inhibited chlorophyll formation in velvetleaf plants. In contrast, metabolites from neither cotton or soybean inhibited chlorophyll formation in soybean. This is clear evidence for active clomazone metabolites in cotton and also suggest that soybean, but not velvetleaf, can detoxify the metabolites. Thus, both activation and detoxification of active metabolites could control clomazone toxicity in plants.

These studies, with the exception of that of Goetz et al. (1988), suggest that clomazone selectivity between clomazone-tolerant and clomazone-susceptible weed and crop species cannot be attributed to differential metabolism or rate of metabolism (Weston and Barrett et al., 1989; Norman et al., 1990a, Scott et al., 1992; Vencill et al., 1990; Weimer et al., 1991).

While clomazone results in bleached plants, the clomazone mode of action has not been identified. Clomazone was reported to inhibit the formation of chlorophyll, carotenoids, plastoquinone, tocopherol, and gibberellins, all of which are dependent on terpenoid biosynthesis, in higher plants including soybean and cotton photomixotropic cell suspension cultures (Norman et al., 1990a). Clomazone also prevented the phytylation of chlorophyllide in cowpea (*Vigna unguiculata*) (Duke & Kenyon, 1986). In green algae (*Scenedesmus*), the phytol pool was much lower in clomazone treated cells (Sandman & Boger, 1986). Treatment of pitted morningglory (*Ipomea lacunose*) (Duke et al.,

1985) or the microalga *Aphanocapsa* (Sandman & Boger, 1987) with clomazone did not result in phytoene or phytofluene accumulation. An increase in isopentenyl pyrophosphate (IPP), which is an intermediate in the isoprenoid pathway, was observed in spinach (*Spinacia oleracea*) extracts treated with clomazone (Sandman & Boger, 1987). Clomazone did not effect IPP isomerase or the biosynthesis of geranylgeranyl pyrophosphate (Croteau, 1992; Weimer et al., 1992, Scott et al., 1994). Also, previously identified potential clomazone metabolites: 2-chloro-benzyl alcohol, 2-chloro-benzyl aldehyde, 2-chlorobenzoic acid, and 2-chloro-4-hydroxy benzoic acid did not inhibit, either in vivo or in vitro, extractible IPP isomerase and prenyl transferase activity (Scott et al., 1994). Taken together, these past studies failed to determine any specific enzymatic target site for clomazone.

At the time these studies were performed, the acetate/mevalonate pathway, proceeding from acetyl CoA, was considered to be the unique pathway for the 5C isoprenoid precursor unit is IPP. In this pathway, mevalonate is synthesized by the condensation of three acetyl CoA and then 3-hydroxy-3-methylglutaryl-CoA. Subsequent phosphorylation and decarboxylation of mevalonate yields IPP (Lichtenthaler, 1999).

Recently, a second IPP biosynthetic pathway, the 1-deoxyxylulose 5-phosphate/methylerythritol 4-phosphate (DOXP/MEP) pathway, which proceeds from glyceraldehyde 3-phosphate (GAP) and pyruvate, not from mevalonate, was discovered (Lichtenthaler, 1999). The first product of this pathway is DOXP produced by DOXP synthase. The second product is MEP, synthesized by DOXP reductoisomerase. The next three steps and the genes for the enzymes involved in the synthesis of IPP were recently characterized (Rohdich et al., 1999; Kuzuyama et al., 2000; Rohdich et al., 2000; Lutgen et al., 2000; Herz et al., 2000). Additional reactions and the enzymes involved are suspected have not been identified yet. These two independent pathways, mevalonate and DOXP/MEP, for IPP and isoprenoid formation work in different cellular compartments and deliver differential isoprenoid end

products. The acetate/mevalonate pathway is localized in the cytosol and synthesizes sterols and sesquiterpenes while the DOXP /MEP pathway is localized in the plastids and synthesizes carotenoids, phytol, the prenyl side-chain(s) of plastoquinone-9 and phylloquinone K, isoprene plus mono and diterpenes (Lichtenthaler 2000).

The first objective of these studies was to determine the basis of safening cotton against clomazone by phorate and the next objective was to find the biochemical target site of clomazone.

in maize microsomes (Baerg et al., 1996). In vitro studies showed phorate and terbufos were weak inhibitors of P450 hydroxylation of nicosulfuron and bentazon in corn (Diehl et al., 1995; Baerg et al., 1996) microsomes. However, terbufos metabolites, particularly the sulfone of terbufos, were strong inhibitors of nicosulfuron metabolism in vivo and in vitro (Diehl et al., 1995). Further work found that the terbufos sulfone (phosphorodithioic acid S[-[[1,1-dimethylethyl]sulfonyl]-methyl]O,O-diethyl ester) inhibited the metabolism of, not only nicosulfuron, but also that of bentazon, chlorimuron ethyl (2-[[[(4-chloro-6-methoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]benzoic acid), imazethapyr, and malathion (Baerg et al., 1996) in maize microsomes. Similarly, thifensulfuron injury to soybeans and corn, when applied with chlorpyrifos and malathion, appears to result from insecticide inhibition of thifensulfuron detoxification (Ahrens and Panaram, 1997). Recently, studies with terbufos pretreated corn plants demonstrated inhibition of rimsulfuron (1-(4,6-dimethoxypyrimidin-2-yl-3-(ethylsulfonyl)-2-pyridylsulfonyl)urea) metabolism (Koeppel et al., 2000).

The objective of this research was to establish how the organophosphate insecticide phorate safens cotton against clomazone.

MATERIALS AND METHODS

Chemicals

[¹⁴C] ring labeled clomazone (28.0 mCi/mmol), technical grade clomazone (98% pure), clomazone metabolites, and formulated material (Command ME) were provided by FMC Corporation (Princeton, NJ, USA). Technical grade phorate was purchased from Chem Service (NJ, USA).

Whole plant tolerance studies

Tolerance of cotton seedlings to clomazone was determined by measuring the effect of clomazone on chlorophyll and carotenoid contents of new leaves. Cotton seeds were planted in plastic trays containing vermiculite and watered

with half strength nutrient solution (Maynard and Barker, 1970) as needed. Plants were grown under continuous light providing $46 \mu\text{E m}^{-2} \text{s}^{-1}$ PAR at 30 C. Plants, 8-10 days old, cotyledon stage, were transferred into a hydroponic system described by Diehl et al.(1995). After one day in nutrient solution, seedlings were transferred into 300 ml of clomazone solutions of 0, 1, 3, 10, 30, 100, 1000 or 3000 nM. The clomazone solutions were prepared using half strength nutrient solution plus formulated clomazone. Seedlings were treated for 6 d. The containers were sealed with parafilm to inhibit clomazone losses through volatility. At the end of the treatment period, new leaves were harvested and weighed. Chlorophyll and carotenoids were quantified by the method described⁴ by Lichtenthaler et al. (1987). Leaves were homogenized in 10 ml of 80% acetone and then the homogenates were centrifuged at 10,000 g for 10 min. Absorbance values of the supernatants at 663.2, 646.8, and 470 nm and the equations of Lichtenthaler et al. (1987) were used to determine total chlorophyll and total carotenoid absorbance values.

The effect of the insecticide phorate on clomazone injury was determined by including 0, 0.5, 5, or 50 μM phorate in the root medium. Phorate was added to the solution in DMSO (1% DMSO by volume in final reaction mixture) for 1 d. To test the critical time for the presence of phorate, phorate was included 1 d before, the same day, or 1 d after 100 nM clomazone had been applied and 3 days (1 day before, same day, 1 day after clomazone).

In addition to the treatments with clomazone, cotton seedlings were also treated with 5-OH clomazone (2-[(2-chlorophenyl)methyl]-5-hydroxy-4,4-dimethyl-3-isoxazolidinone) at 100, 1000, or 10 000 nM. In addition, the seedlings were treated with or without phorate (50 μM) with a 1000 nM 5-OH clomazone concentration as described above.

Chlorophyll and carotenoid reductions were determined by comparison to values of the untreated check. The experiment used a completely random design with each assay being repeated at least two times and each treatment

was replicated 3 times. Data were analyzed by ANOVA (SAS) at 5% level of probability.

Effect of phorate on in vivo clomazone metabolism

Cotton seedlings were grown in vermiculite as for the whole plant studies and harvested 8-10 d after planting. In the afternoon prior to harvest, the vermiculite was saturated with water, the plants covered with plastic, and the tray was placed into a dark incubator at 30 C overnight. The following morning, the cotton plants were removed from the vermiculite and the roots were washed with distilled water. The roots were placed into a solution with 0.45 μCi [^{14}C] ring labeled clomazone plus 0.1 μM technical clomazone with and without 50 μM phorate added in DMSO (1% DMSO by volume in final reaction mixture). Plants were then removed from the treatment solution and the roots placed into distilled water. The plants were kept for 16h at 27C in a dark incubator. The roots of harvested plants were then washed with distilled water and plants separated into root and shoot tissue. The plant tissue was ground to a fine powder with a mortar and pestle in the presence of liquid nitrogen, mixed with 10 ml of water, and then centrifuged at 10,000 g for 10 min. The supernatant (aqueous extract) was removed and pellet suspended twice with 5 ml of methanol and the suspension was again centrifuged. The supernatant was again removed (methanol extract). The aqueous extract and then methanol extract were loaded sequentially onto a C18 Sep-Pac column (P463R, PrepSep R-C¹⁸, Fisher Scientific, Fair Lawn, NJ). The eluants after loading the aqueous and methanol extracts were collected and kept separate. The column was washed with 5 ml methanol and the flow through again collected. ^{14}C associated with the aqueous flow-through and the methanol flow-through plus methanol wash was determined by means of liquid scintillation spectrometry (LSS). Unextracted radioactivity was determined by oxidizing the pellet in a biological sample oxidizer (Model 307, Packard Instrument Company, Meriden, CT). The released $^{14}\text{CO}_2$ was trapped and

counted by LSS. The fraction of the total recovered ^{14}C in the methanol, water, and pellet was calculated.

In a second study, excised cotton shoots were used instead of whole plants to determine the effect of phorate on in vivo clomazone metabolism. Cotton plants were grown as described above to the same age for the whole plant study. Shoots were harvested by cutting above the vermiculite surface, and then placing the cut ends of the shoots under water and then recutting them below the surface. Incubations of shoots, extraction of radioactivity, determination of counts in each fraction, and analysis of data were done as described above.

The experiment used a completely random design with each assay being repeated at least two times and each treatment was replicated 3 times. Data were analyzed by ANOVA (SAS) at 5% level of probability.

HPLC separation of clomazone and metabolites

Separation and identification of clomazone and metabolites in the extracts from the shoot tissue were performed utilizing an HPLC (high-performance liquid chromatography) equipped with a Whatman-Partisal 5 ODS-3 4.6×250 mm column. ^{14}C in the eluate was detected with a Packard Instruments Radiomatic Flo-One A500 series detector. HPLC conditions for analysis of clomazone and metabolites were: flow of 1.5 ml min^{-1} with a gradient from 25 to 60% acetonitrile (ACN) over 0 to 45 min. Retention times of radiolabelled peaks were compared to retention times of analytic standards detected by monitoring the column eluent at 218 nm. To hydrolyze any conjugates present, the water extracts were hydrolyzed with 1N HCl solution at 80 C for 2 h. The mixture was neutralized with 200 μl of 1N NaOH. Enzymatic hydrolysis of the water extract was done with 100 mg β -glucosidase (Sigma Chemicals) in a 0.1 M, 100 ml sodium acetate buffer (pH 5.0) at 37 C for 4 h followed by a second 4 h incubation done after the addition of another 100 mg of β -glucosidase. The hydrolyzed extracts were analyzed with HPLC as described above. The

percentage of total ^{14}C present in each peak was calculated as a percentage of total ^{14}C recovered in the water fraction.

Each assay being repeated at least two times and each treatment was replicated 3 times. Data were analyzed by ANOVA (SAS) at 5% level of probability.

RESULTS

Whole plant tolerance studies

As expected, clomazone reduced both chlorophyll and carotenoid levels in new leaves (Figure 2.1). A 1000 nM clomazone concentration reduced total chlorophyll 90% and total carotenoids 79% 6 d after treatment. However, a 10 nM clomazone concentration reduced chlorophyll and carotenoids 73% and 61 % respectively and 1 nM clomazone reduced chlorophyll and carotenoid concentrations less than 20%.

Inclusion of 0.5, 5, or 50 μM phorate in solution with clomazone (100 nM) partially prevented the loss of total chlorophyll and carotenoids in a rate dependent manner (Figure 2.2.). The greatest safening occurred with 50 μM phorate.

In a study to examine the timing of safening from a one day clomazone (100 nM) treatment, maximum safening was achieved when phorate (50 μM) was applied the same day as clomazone. Less safening was observed with phorate the day before or after clomazone. Inclusion of phorate in solution for three days (day before, day of treatment, day after) did not improve safening (Figure 2.3).

Treatment of 7-d-old cotton seedlings with 5-OH clomazone decreased chlorophyll and carotenoid content in a rate dependent manner as clomazone (Figure 2.4). A 100 nM 5-OH clomazone concentration reduced total chlorophyll and total carotenoids 11%. In contrast, 1 nM clomazone reduced

total chlorophyll and total carotenoid rate approximately 8 and 9 times more than 100 nM 5-OH clomazone concentration. Increasing the 5-OH clomazone from 1000 nM to 10000 nM only slightly increased inhibition of both chlorophyll and carotenoids. The 76 % reduction of both total chlorophyll and total carotenoids by 10 000 nM 5-OH clomazone equivalent to the reduction by 100 and 1000 nM clomazone concentrations. Clomazone was approximately 10 to 100 times more toxic than 5-OH clomazone.

Phorate (50 μ M) did not prevent injury of the 7-d-old cotton seedlings treated with 1000 nM 5-OH clomazone (Table 2.1). However, with the same phorate concentration, cotton injury from clomazone (100 nM) was reduced significantly (2.2).

Effect of phorate on in vivo clomazone metabolism

HPLC analysis of the extracts showed the methanol extract contained only clomazone while the aqueous extract contained only clomazone metabolites (data not shown). Therefore, the fraction of the recovered radioactivity in the methanol fraction was considered to represent unmetabolized clomazone while the aqueous extract was considered to represent metabolites. Phorate reduced clomazone metabolism in the shoot tissue but not in the root tissue (Table 2.2). The unextracted radioactivity did not change in both shoot and root tissue in the presence of phorate.

Similarly, phorate reduced clomazone metabolism in excised cotton shoots (Table 2.3). The amount of clomazone metabolites formed in excised cotton shoots (83% of the recovered radioactivity) was higher than that in intact plants (42%) (Table 2.2 and Table 2.3). However, in both cases, phorate reduced the formation of clomazone metabolites.

HPLC separation of clomazone and metabolites

Retention times of authentic standards of clomazone and the potential clomazone metabolite isoxazolidinone (4,4-dimethyl-3-isoxazolidinone), 4'-5

diOH clomazone (2-(2-chloro,4'-hydroxyphenyl)methyl-5-hydroxy-4,4-dimethyl-3-isoxazolidinone), 2-chloro benzyl alcohol, 5-OH clomazone, 5-keto clomazone (2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3,5-isoxazolidinedione) were 34, 5, 11, 15, 23, and 36 min, respectively (data not shown). HPLC analysis of clomazone metabolites in the aqueous fraction of the extracts showed no clear reduction in any single metabolite in response to phorate treatment (Table 2.4). The most polar clomazone metabolites at 3-8 min increased between phorate treatments and in which hydrolysis treatments (unhydrolyzed, HCl, β -glucosidase) (Table 2.4). These possibly represent all the breakdown products of clomazone that have similar structures as isoxazolidinone (Elnaggar, 1992). Phorate reduced the peaks at 13 min in the unhydrolyzed and β -glucosidase treatments and the peaks at 17, 21, and 23 min in the β -glucosidase treatment. These are possible glucosides and glucose conjugates of clomazone produced by cotton. They constitute the majority of the clomazone metabolites. The peak at 11 min disappeared following HCl or β -glucosidase treatment suggesting it is a glucoside of a clomazone metabolite. Since the conjugates of herbicides are usually non-phytotoxic (Shimabukuro, 1985), these are unlikely to be the cause of the herbicidal activity of clomazone. The peaks at 31 and 36 minutes were slightly changed between hydrolysis treatments. The peak at 36 min cochromatographed with 5-keto clomazone, the least polar metabolite treatment. it was reduced slightly in response to phorate treatment following the HCl hydrolysis.

DISCUSSION

Phorate present at the same time as clomazone reduced the visual injury (bleaching) (data not shown) and the inhibition of chlorophyll and carotenoid formation by the herbicide (Table 2.1). These results are in agreement with previous field studies where less cotton injury was noted when clomazone was applied in combination with disulfoton or phorate (York et al., 1991). Our

studies are also consistent with recent studies where clomazone metabolism in cotton was reduced by phorate and disulfoton (Culpepper et al., 2001). These results suggest a clomazone metabolite(s), and not clomazone, may be the phytotoxic agent in cotton and phorate and disulfoton are preventing its formation. Further evidence for clomazone bioactivation was obtained from other studies in our laboratory with corn (Avduishko, unpublished). Phorate was shown to protect corn from clomazone injury in these studies and to reduce clomazone metabolism in the corn plants. In addition to phorate, the known P450 inhibitors ABT (1-aminobenzotriazol), malathion, phenobarbital, tetracycline (5-(4-chlorophenyl) 3,4,5,9,10-pentaaza-tetracyclo[5,4,10,2,6,0,8,11]dodeca-3,9-diene), terbufos sulfone, paclobutrazol ((2RS, 3RS)-1-(4-chlorophenyl)-4, 3-dimethyl-2- (1H-1, 2, 4-triazol-1-yl) pentan-3-ol), plus verbutin (dibenzo-1,4-thiazine) safened the corn from clomazone metabolism. These also inhibited metabolism of clomazone in corn. This clearly showed that inhibition of P450(s) activity was critical to the safening phenomenon. Additionally, these P450 inhibitors and verbutin also safened cotton from clomazone. Studies with microsomes prepared from corn seedlings grown from naphthalic anhydride treated seed demonstrated phorate inhibited the P450 catalyzed formation of a single product which co-chromatographed with 5-OH clomazone. This led to the focus on 5-OH clomazone in cotton. This was previously found to be the one of the major clomazone metabolites in soybean (EINaggar, 1992). This metabolite was seen in vivo at very low levels in metabolism studies with cotton shoots (Table 2.4) and, similar to phorate, other P450 inhibitors reduced its formation in corn microsomes (Ferhatoglu et al., 2000). Thus, corn microsome activity for 5-OH clomazone formation may correspond to clomazone hydroxylase activity seen in vivo in cotton. Organophosphate insecticides, including phorate, have been recognized as inhibitors of cytochrome P450 monooxygenases in plants (Baerg et al., 1996; Diehl et al., 1995; Kreuz and Pfister, 1992). Although the metabolic pathway of clomazone in cotton has not been established, initial

metabolites of clomazone in soybean were all hydroxylated derivatives (ElNaggar et al., 1992) suggesting P450 may be involved in clomazone metabolism.

Herbicidal activity (bleaching) from the clomazone metabolites 5-OH-clomazone and 5-keto clomazone was observed in cotton (data not shown) and in some other crop plants (Chang et al., 1987). A clomazone metabolic pathway leading to formation of 5-OH clomazone and then 5-keto clomazone is present in soybean (El Naggar et al., 1992). Although phorate was effective in safening cotton from clomazone, phorate is ineffective as a safener for 5-OH clomazone. Whether 5-OH clomazone and/or 5-keto clomazone or other clomazone metabolites arising from them is the actual toxicant cannot be determined from these studies.

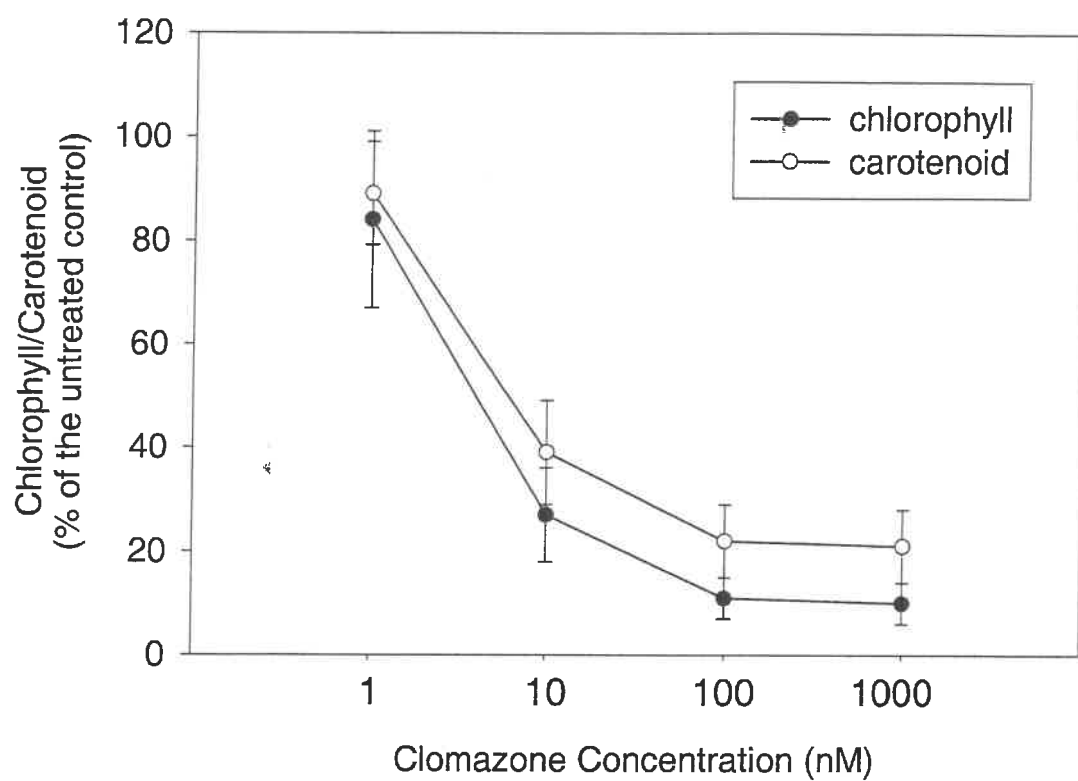


Figure 2.1: Effect of clomazone concentrations on the total chlorophyll and carotenoid content levels in new leaves of 7- d-old cotton seedlings.

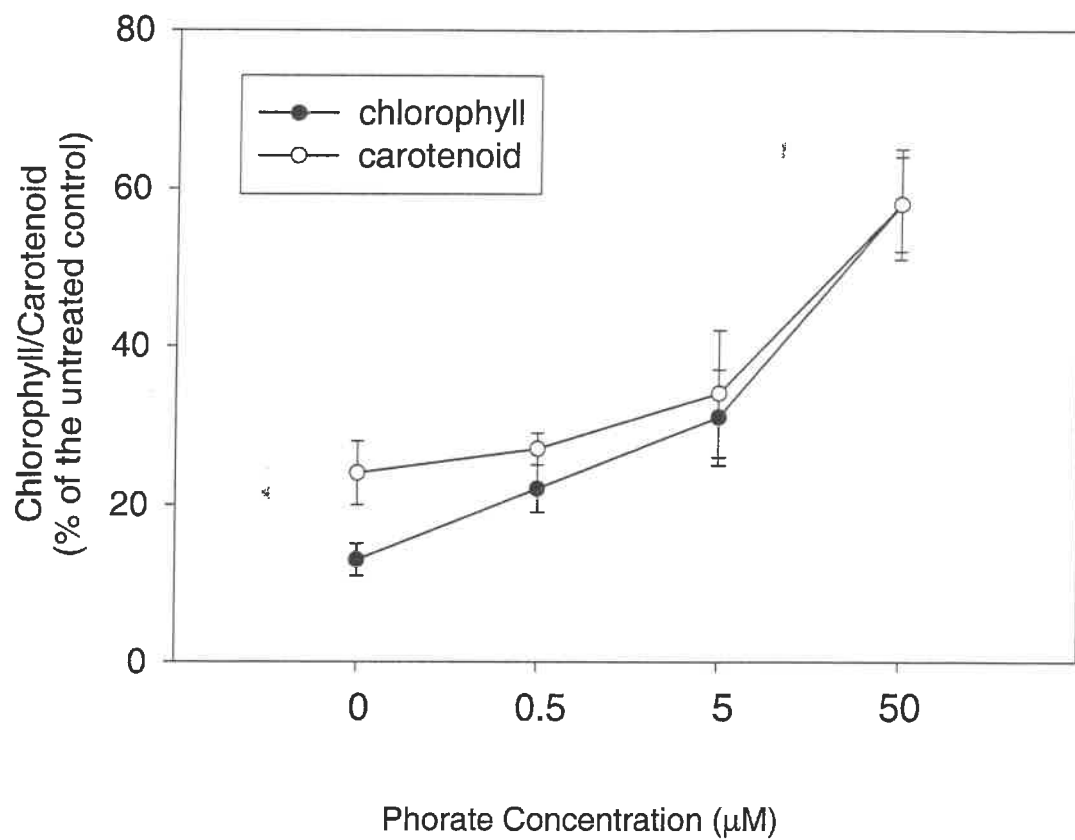


Figure 2.2: Effect of phorate on the total levels of chlorophyll and carotenoids in new leaves of 7- d-old cotton seedlings treated with 100 nM clomazone.

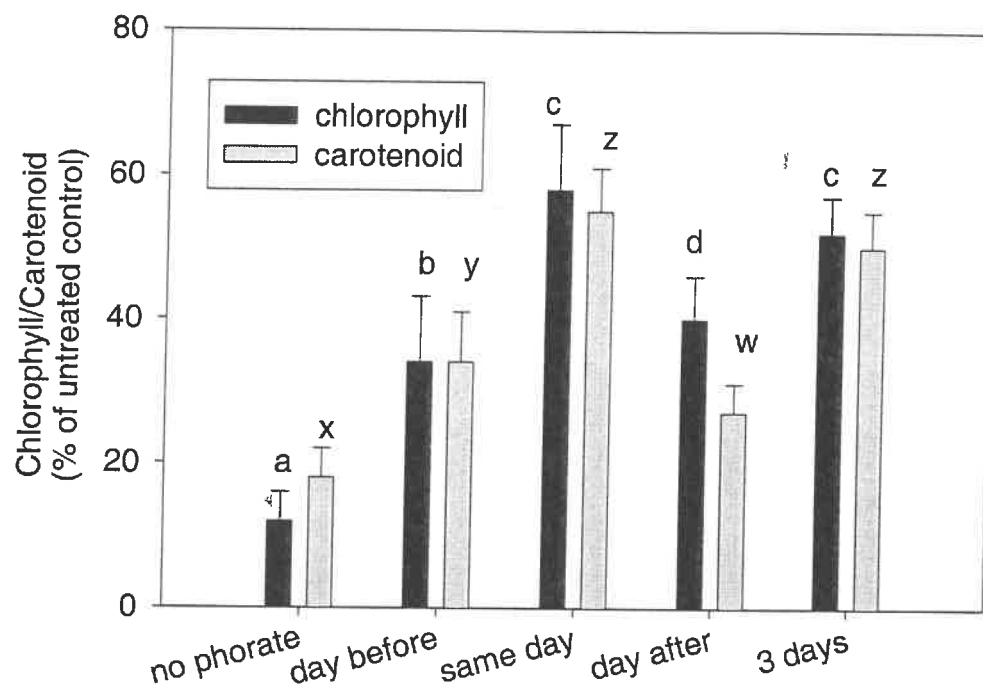


Figure 2.3: Effect of phorate (50 μ M) treatment timing in relation to clomazone (100 nM) treatment on the total chlorophyll and carotenoid content levels in new leaves of 7- d-old cotton seedlings. Vertical bars represent the mean $\pm$ SD. Treatments with the different letters within the chlorophyll or carotenoids are significantly different ($P < 0.05$).

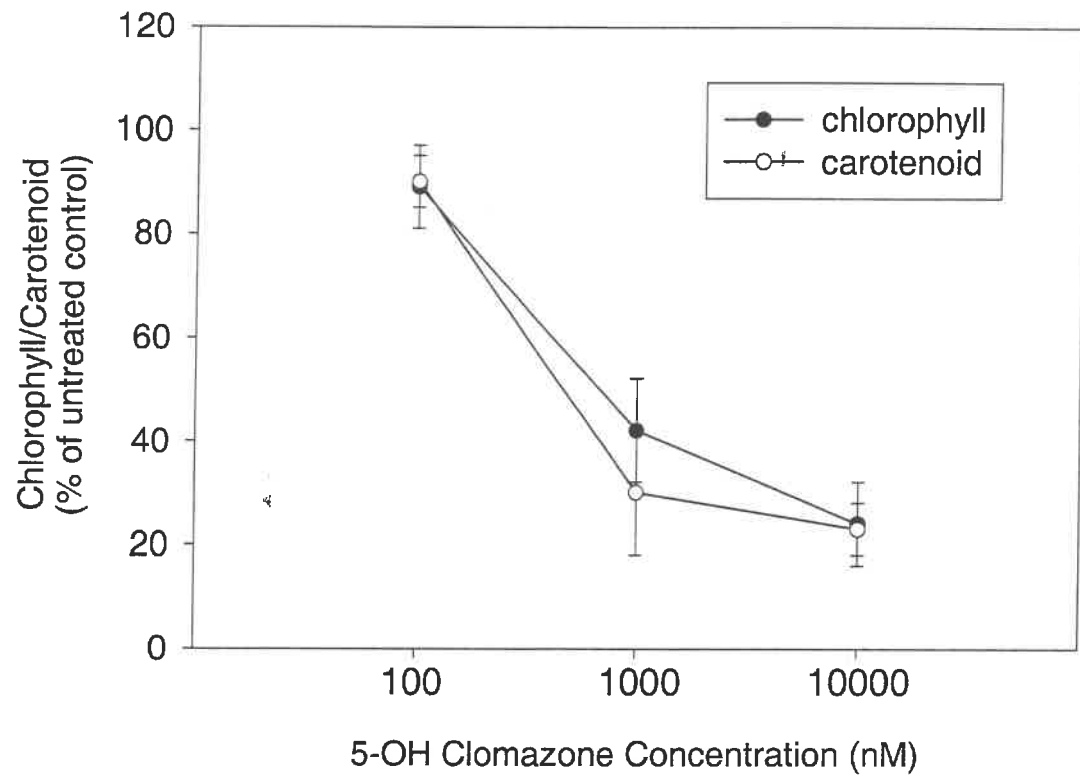


Figure 2.4: Effect of 5-OH clomazone on the total chlorophyll and carotenoid content levels in new leaves of 7- d-old cotton seedlings.

Table 2.1: Effect of 50 μ M phorate on the total chlorophyll and carotenoid content levels in new leaves of 7- d-old cotton seedlings treated with 1000 nM 5-OH clomazone .

Treatment	Chlorophyll ^a	Carotenoid ^a
% of untreated control		
None	56 $\pm$ 13	57 $\pm$ 20
+Phorate	67 $\pm$ 7	65 $\pm$ 9

^a Mean $\pm$ SD.

Table 2.2: Effect of 50 μ M phorate on the radioactivity (% of total recovered) associated with clomazone and metabolites plus that unextracted 24 h after ^{14} C-clomazone treatment of 7-d-old intact cotton plants

Tissue	Phorate	Clomazone ^a	Metabolites ^a	Unextracted ^a
Radioactivity (% of total recovered)				
Shoot	-	54 $\pm$ 2	42 $\pm$ 2	9 $\pm$ 4
Shoot	+	71 $\pm$ 1 *	27 $\pm$ 10 *	9 $\pm$ 4
Root	-	45 $\pm$ 4	55 $\pm$ 4	12 $\pm$ 3
Root	+	44 $\pm$ 8	56 $\pm$ 8	14 $\pm$ 2

^aMean $\pm$ SD

* Indicates significant difference ($P < 0.05$) compared to control (no phorate) within same tissue and within same column.

Table 2.3: Effect of 50 μ M phorate on radioactivity (% of total recovered) associated with clomazone and clomazone metabolites plus that unextracted 24 h after ^{14} C-clomazone treatment of excised 7-d-old cotton shoots.

Phorate	Clomazone ^a	Metabolites ^a	Unextracted ^a
Radioactivity (% of total recovered)			
-	9 $\pm$ 2	83 $\pm$ 3	8 $\pm$ 3
+	22 $\pm$ 3 *	72 $\pm$ 3 *	6 $\pm$ 6

^aMean $\pm$ SD

* Indicates significant difference ($P < 0.05$) compared to control (no phorate) within same column.

Table 2.4: Effect of 50 μ M phorate on distribution of ^{14}C associated with peaks in aqueous extracts 24 h after ^{14}C -clomazone treatment of 7-d-old cotton shoots.

Hydrolysis Treatment	Phorate Treatment	Distribution of ^{14}C (% total peaks)							
		Elution Time (min)							
		3-8	11	13	17	21	23	31	36
Unhydrolyzed	None	41*	11	11*	7	<1	<1	<1	^A ND
	+Phorate	65	12	5	7	<1	<1	<1	^A ND
HCl	None	25*	^A ND	7	8	8	1	2	2
	+Phorate	54	^A ND	6	5	6	1	<1	<1
β - glucosidase	None	42*	^A ND	5*	21*	8*	3*	^A ND	^A ND
	+Phorate	70	^A ND	1	12	5	<1	^A ND	^A ND

^AND=not detected

* Indicates significant difference ($P < 0.05$) between phorate treatments within a hydrolysis and within the same column.

Chapter Three

Studies of the Clomazone Mode of Action

INTRODUCTION

Clomazone (2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone) is a selective soil-applied herbicide currently registered for use in pepper (*Capsicum annum*), pumpkin (*Cucurbita pepo*), soybean (*Glycine max.*), cotton (*Gossypium hirsutum.*), tobacco (*Nicotiana tabacum*), and pea (*Pisum sativum*). Clomazone controls many annual and broadleaf weeds (Blackshaw and Esua 1991; Jordan et al., 1993; Langton et al., 1997; Westburg et al., 1989). Clomazone was reported to interfere with chloroplast development and to reduce or prevent accumulation of plastid pigments in susceptible species (Duke et al., 1985). Clomazone treatment causes white, yellow, or pale-green plants, depending on the species and/or method and dose of treatment (Duke & Kenyon, 1986). Clomazone inhibits the formation of chloroplast bound isoprenoids including the diterpenic phytyl chain of chlorophyll (Duke et al., 1985; Duke & Kenyon, 1986; Goetz et al., 1988; Vencill et al., 1989; Norman et al, 1990a, Norman et al, 1990 b; Scott et al., 1992, 1994), carotenoids (tetraterpene; 20C) (Duke & Kenyon, 1986; Norman et al., 1990a, Norman et al, 1990b; Duke et al., 1991; Scott et al., 1992, 1994), plastoquinone (45C) (Norman et al., 1990a), tocopherol (45C) (Norman et al., 1990b), and gibberellins (Vencill et al., 1989) in higher plants and in *Scenedesmus acutus* algae (Sandman & Boger, 1986). When clomazone was applied to etiolated plants, the initial effect, upon placing the plants into the light, is a retardation of chloroplast development. Disruption of chloroplast membranes occurred only after 12 to 24 h (Duke & Paul, 1986). Clomazone had no effect on protochlorophyllide levels nor did it influence phototransformation of protochlorophyllide to chlorophyllide in both cowpea (*Vigna unguicula*) and pitted morninglory (Duke & Kenyon, 1986; Duke et al., 1985). However, the shibata shift, an in vivo spectral shift of

protochlorophyllide absorption is greatly slowed in both cowpea (Duke & Paul, 1986) and pitted morninglory (Duke et al., 1985). The shibata shift is thought to be due to phytylation of chlorophyllide (Henningesen & Thorne, 1974) leading to speculation that clomazone inhibits synthesis of phytol, a diterpenoid (10C) (Duke & Kenyon, 1986). Later studies found the phytol pool was much lower in *Scenedesmus* cells treated with clomazone (Sandman & Boger, 1986). Retardation of longitudinal growth in both light and darkness by clomazone led to speculation that the herbicide inhibited synthesis of gibberellic acid, another diterpenoid. The growth-inhibiting effect of clomazone on pea (Sandman & Boger, 1986) and corn (*Zea mays*) (Vencill et al., 1989) seedlings can be partially reversed with exogenous gibberellic acid treatments.

All previous herbicides known to inhibit carotenoid biosynthesis block phytoene desaturase or later steps in the synthesis of carotenoids (Duke, 1985; Ridley, 1982) causing abnormal accumulation of phytoene and/or phytofluene. Treatment of *Scenedesmus* algae (Sandman & Boger, 1986), pitted morninglory (Duke et al., 1985), and cotton (Duke et al., 1991) with clomazone did not cause phytoene or phytofluene accumulation.

Further studies demonstrated that clomazone inhibited synthesis of geranylgeranyl pyrophosphate in a cell-free spinach preparation (Sandman & Boger, 1987) but increased isopentenyl pyrophosphate (IPP) levels. It was hypothesized that IPP isomerase or a prenyl transferase is inhibited by clomazone. Prenyl transferases are required for each successive addition of a 5C unit (IPP) to simpler terpenoids. In studies with daffodil (*Narcissus* sp.) chromoplast and mustard (*Sinapis alba*) etioplasts, clomazone did not inhibit the activity of IPP isomerase, prenyl transferases, or phytoene synthase, even at higher clomazone concentrations that cause loss of carotenoids in intact plants (Lutzov et al., 1990). Weimer et al., (1992) found that clomazone did not affect the synthesis of IPP from mevalonate or conversion of IPP to geranylgeraniol derivatives (geraniol, farnesol, phytol) in osmotically shocked spinach chloroplasts. Clomazone did not have a direct effect on IPP isomerase

or the biosynthesis of geranylgeranyl pyrophosphate (Croteau, 1992; Weimer et al., 1992, Scott et al., 1994). In further studies, the sesquiterpenoid hemigossypol and the dimeric sesquiterpenoid gossypol accumulated in clomazone treated cotton (*Gossypium hirsutum* L.Z) (Duke et al., 1991). These compounds are derived from farnesyl pyrophosphate, the fifteen carbon immediate precursor of geranylgeranyl pyrophosphate. Clomazone had no effect on the in vitro activity of hydroxy methylglutaryl-coenzyme A reductase (HMGR) (Ji & Hatzios, 1990), the enzyme that synthesizes mevalonate and which is a substrate for isopentenyl pyrophosphate isomerase, feedback regulated HMGR is stimulated 4-7 fold by clomazone treatment of 5-d-old light grown maize seedlings.

Due to an inability to identify a clomazone site of action in previous studies, bioactivation of clomazone was suspected. The most significant evidence for bioactivation was provided in studies where clomazone metabolites extracted from cotton (less clomazone tolerant) cell culture were toxic to velvetleaf (clomazone sensitive species). However, clomazone metabolites extracted from soybean (clomazone tolerant species) cell cultures were not toxic to velvetleaf (Norman et al., 1990b). Additionally, clomazone metabolites from both cotton and soybean cell cultures did not cause toxicity in soybean. There was no difference in the amount of clomazone metabolism between soybean and cotton cell cultures 48 hours after treatment.

In further studies, clomazone and the potential clomazone metabolites were evaluated for possible inhibition of IPP isomerase and prenyl transferase activity. The clomazone metabolites 2-chloro-benzyl alcohol, 2-chloro-benzyl aldehyde, 2-chlorobenzoic acid, 2-chloro-4-hydroxy benzoic acid or clomazone did affect IPP isomerase and prenyl transferase activity from tomato (extremely clomazone sensitive) or tobacco (extremely clomazone tolerant) cell suspension cultures or in light or dark grown tomato or pepper cotyledons (Scott et al., 1994).

At the time these previous studies were done, it was accepted that IPP, the common precursor of all isoprenoids, was synthesized through the well known acetate/mevalonate pathway. In this pathway, three acetyl CoA molecules form 3-hydroxy-3-methylglutaryl-CoA, and then mevalonate. Subsequent phosphorylation and decarboxylation of mevalonate yield IPP (Lichtenthaler, 1999). However, a second IPP biosynthetic pathway, the 1-deoxyxylulose 5-phosphate/methylerythritol 4-phosphate (DOXP/MEP) pathway, which proceeds from glyceraldehydes 3-phosphate (GAP) and pyruvate, rather than mevalonate, was later discovered (Lichtenthaler, 1999). DOXP and MEP are the first two intermediates and are formed by DOXP synthase and DOXP reductoisomerase, (Takahashi et al., 1998; Lange & Croteau, 1999), respectively, of the DOXP/MEP pathway. The next three steps and the genes for the responsible enzymes of the DOXP/MEP pathway were recently characterized (Rohdich et al., 1999; Kuzuyama et al., 2000; Rohdich et al., 2000; Lutgen et al., 2000; Herz et al., 2000). Fosmidomycin (phosphonic acid, [3-(formylhydroxyamino)propyl]), an anti-malaria drug (Jomaa et al., 1999), inhibits the second step (DOXP reductoisomerase) in the DOXP/MEP pathway (Zeidler et al., 1998; Schwender et al., 1999; Mueller et al., 2000). The DOXP/MEP pathway produces isoprenoids used for carotenoid, phytol, plastoquinone-9, isoprene, mono- and diterpene (Ginko and Taxus (Einsenreich et al., 1996)) biosynthesis in the plastid (Lichtenthaler, 1997; Lichtenthaler, 1999). The cytoplasmic acetate/mevalonate pathway produces sterols, sesquiterpenes, and triterpenoids (Lichtenthaler et al., 1997a; Lichtenthaler, 1999). The DOXP/MEP pathway occurs in plants (Lichtenthaler et al., 1997, Zeidler et al., 1997) and bacteria (Rohmer et al., 1993, Lichtenthaler, 1999, Rohmer, 1998, Jomaa et al., 1999).

Chloroplasts appear to be an autonomous compartment for IPP biosynthesis (Lichtenthaler, 1997) but there is evidence for a limited exchange of isoprenoids and precursors between plastids and the cytoplasm. Mature chloroplasts may be dependent on cytosolic IPP formation because plastidic

IPP biosynthesis in barley has its maximum activity at the leaf base (containing developing chloroplasts) and almost no activity at the leaf tip (containing mature chloroplasts) (Heintze et al., 1990). The ability of the chloroplast to import IPP from the extraplastic space gradually increases to substitute for the loss of an endogenous intermediate supply for chloroplast isoprenoid synthesis (Heintze et al., 1990).

The failure to identify a site of clomazone action in previous studies may be due to the focus on inhibition of the cytoplasmic acetate/mevalonate pathway, rather than the plastidic DOXP/MEP pathway. In addition, a metabolite of clomazone, rather than the parent clomazone molecule, may be the active inhibitor.⁴

Our hypothesis was that a clomazone metabolite is the active inhibitor. Support for clomazone bioactivation is found in previous studies examining relative clomazone metabolism in sensitive and tolerant species and studies of clomazone safening by organophosphate insecticides. Generally, these studies show that less conversion of clomazone to metabolites was associated with greater clomazone tolerance. Data support clomazone bioactivation in cotton (Norman et al., 1990b). Phorate protects the cotton from bleaching (Harrison et al., 1992; Hayes et al., 1993, Ferhatoglu et al., 1999) and reduces clomazone metabolism in cotton (Ferhatoglu et al., 2000; Culpepper et al., (2001). It was previously shown that clomazone is converted to typical P450 associated metabolites in soybean (OH-derivatives, EINaggar et al., 1992). Although the metabolic pathway of clomazone in cotton has not been published, the clomazone metabolic pathway in soybean suggests that P450(s) could metabolize the herbicide in cotton. Phorate is known as an inhibitor of P450 reactions (Baerg et. al., 1996, Diehl et al., 1995). Phorate and other known P450 inhibitors reduce the formation of a specific clomazone metabolite that cochromatographed with 5-OH clomazone in corn microsomes (Ferhatoglu et al., 2000). Additionally, 5-OH clomazone is thought to lead to 5-keto clomazone in soybean (EINaggar et al., 1992). While our studies were

underway, a report was published indicating that 5-keto clomazone inhibited DOXP synthase (Mueller et al., 2000). Therefore, our objective was to determine if clomazone, 5-OH clomazone, and/or 5-keto clomazone inhibited the DOXP/MEP pathway.

MATERIAL and METHODS

Inhibition of Isoprenoid Biosynthesis

Isolation of chloroplasts

Spinach [(*Spinacia oleracea* var. *Bloomsdale* (Turner Seed Inc.)] was grown in commercial potting mix (Carolina's Choice from Carolina Soil Co.) with a daylength of 14 h and at temperatures of 25°C day and 21°C night in a greenhouse and watered and fertilized as needed. When seedlings were 5 to 15 days old, intact chloroplasts were isolated from fresh young spinach leaves with a one step percoll gradient (Mills and Joy, 1980). The extraction medium included 330 mM sorbitol, 50 mM Tricine-KOH (pH 7.9), 2 mM EDTA, and 1 mM MgCl₂. The leaves were ground with a polytron and the homogenate filtered through 3 layers of washed cheesecloth and 2 layers of miracloth. The filtrate was layered on top of 4 to 6 ml of 40% percoll underlay solution prepared with 330 mM sorbitol, 50 mM Tricine-KOH (pH 7.9). The preparation was then centrifuged at 2500 g for 5 min in a swinging bucket (Sorvall HB-4). The upper phase was aspirated and the pellet resuspended with 300 µL of resuspension buffer (50 mM HEPES (pH 7.9), 1 mM DTT, and 300 mM sorbitol). All isolation steps were performed at 4°C.

Inhibition of isoprenoid biosynthesis in intact chloroplasts.

Chloroplasts (equivalent to 0.4 mg of protein) were incubated in a total volume of 0.5 ml hypotonic incubation buffer (Fellermeier et al., 1999) containing 100 mM HEPES (pH 8), 2 mM MnCl₂, 2mM NADP, 20 µM FAD, 5 mM NAF, 6 mM ATP, 1 mM NADPH and either 16.8 µM [4-¹⁴C] isopentenyl pyrophosphate

(IPP, 57.5 $\mu\text{Ci } \mu\text{mol}^{-1}$) or 114 μM [$2\text{-}^{14}\text{C}$] pyruvate (17.5 $\mu\text{Ci } \mu\text{mol}^{-1}$) at 30°C for 1 h. A 330 mM sorbitol concentration for IPP or a 0.165 mM sorbitol concentration for pyruvate incubation provided hypotonic conditions. The incubations were placed under growth lights (46 $\mu\text{E m}^{-2} \text{s}^{-1}$ PAR) and agitated gently (30 cycle min^{-1}). Following the incubation period, the mixture was extracted twice with 1 ml of chloroform:MeOH (2:1, v/v) and the organic and aqueous layers separated. The organic phase was dried under N_2 and then redissolved with 0.1 ml of ACN:MeOH:2-propanol (85:10:5, v:v:v). Radioactivity associated with both the organic phase and the aqueous phase was quantified by liquid scintillation spectrophotometry. Radioactive compounds in the organic phase were separated and quantified using HPLC equipped with a Partisil 5 ODS-2 (5U; 4.6X250 mm) column and eluted with 100 % ACN:MeOH:2-propanol (85:10:5, v/v) at a flow rate of 1 ml/min (Duke, 1985). Radioactive compounds in the aqueous phase were separated with ion pair HPLC system using an Alltech Adsorbosphere HS column (5U; 250X4.6 mm, Alltech Associates) (Mc Caskill & Croteau, 1993). The aqueous components were eluted using a ternary mobile phase consisting of 10 mM tetra-n-butyl ammonium acetate (TBAA) in water (solvent A), 10 mM TBAA in 70% (v/v) methanol (solvent B), and 70% methanol (solvent C). The elution program consisted of isocratic flow of 100% solvent A for 10 min, a linear gradient to 80% solvent B:20% solvent A over the next 65 min, a linear gradient to 100% solvent C over the next 10 min, and an isocratic flow of 100% solvent C for the next 10 min with a flow rate of 0.8 ml/min. A linear gradient to 100% solvent A the next 5 min and an isocratic flow of 100% solvent A for the next 20 min. The total elution time was 125 min.

Clomazone, 5-OH clomazone, 5-keto clomazone, clomazone, norflurazone (4-chloro-5-methylamino-2-[3-(trifluoromethyl)phenyl]-3(2H)-pyridazinone), or fosmidomycin, all at 20 μM , were preincubated in the reaction mixture for 15 min before addition of ^{14}C -IPP or ^{14}C -pyruvate. Inhibitors, other than fosmidomycin, were added in ethanol to a final ethanol

concentration of 0.1% by volume. The control treatment had ethanol added to the same concentration. Fosmidomycin treatment did not contain ethanol and a treatment without ethanol was used as the fosmidomycin control.

The experiment used a completely randomized design with each assay being repeated at least two times and each treatment was replicated 3 times.

Inhibition of heterologous expressed *Catharanthus roseus* DOXP synthase activity

E. coli expressing *Catharanthus roseus* DXP synthase clone (XL1-blue/pTCRDXS) (Chahed et al., 2000) was provided by Dr. Marc Clastre of the Laboratoire de Biologie Moléculaire et Biochimie Végétale, Faculté de Pharmacie, 31, avenue Monge, 37200 Tours, France. Bacterial cultures were grown at 37°C for 6 h in Luria Bertani medium containing 50 µg mL⁻¹ ampicillin before induction with isopropylthiogalactoside (IPTG, 0.5 mM) at 25°C overnight. Following induction, cells were harvested, and a crude enzyme extract prepared and used to measure DOXP synthase activity as described by Chahed et al. (2000). DOXP synthase assays were performed with extracts from induced *E. coli* cells both expressing and not expressing (vector without insert) *C. roseus* DOXP synthase. The assay system for DOXP synthase consisted of 50 mM Tris-HCl (pH 7.6), 1 mM DTT, 5 mM MgCl₂, 0.5 mM thiamine-diphosphate, 5 mM D-glyceraldehyde 3-phosphate (GAP), 5 mM [2-¹⁴C]-pyruvic acid (37 kBq) and enzyme extract (equivalent to 0.5 mg of protein) in a total volume of 100 µL. GAP was omitted from control treatments. The mixture was incubated for 2 h at 37°C and then the reaction was stopped by heating the mixture at 80°C for 3 min. After centrifugation at 12,000 g for 5 min, ¹⁴C-products in the supernatant were quantified with an ion pair HPLC system as described for the chloroplast assays. Enzyme activity was expressed as pmol of deoxyxylulose synthesized per min per mg of protein. 5-OH clomazone, 5-keto clomazone and clomazone treatment solutions were prepared in ethanol and added to the assays to a final 20 µM concentration.

Final ethanol concentration was 0.5% by volume in all treatments including the control. Assays were preincubated for 15 min before addition of substrates (^{14}C -IPP or ^{14}C -pyruvate). Inhibition of *C. roseus* DOXP synthase activity was determined by comparing the product formed and not formed from non-expressing cells. Solvent concentration had no effect on the product formation. DOXP synthase enzyme activity was calculated in $\text{pmol min}^{-1} \text{mg}^{-1}$ protein. The experiment was repeated two times with three replicates of each treatment. Enzyme activity data were analyzed by ANOVA (SAS).

RESULTS

Peaks of ^{14}C -products formed from ^{14}C -IPP feeding to intact chloroplasts and partitioning into the organic phase were separated by HPLC (Figure 3.1). Clomazone and the clomazone metabolites 5-OH clomazone and 5-keto clomazone did not affect production of these products. The peak eluting at 12 min cochromatographed with an authentic standard of eluted lutein. The other peaks were not identified. A new peak eluting at 26 min and not present in the control sample was detected in the organic phase from the norflurazone treatment. This peak cochromatographed with phytoene standard. Accumulation of phytoene (Mayer et al. 1989) demonstrated an active carotenoid biosynthesis from IPP in the chloroplasts. Five ^{14}C -products from the organic phase from ^{14}C -pyruvate fed chloroplasts were separated by HPLC (Figure 3.2). Neither norflurazone nor clomazone and 5-OH clomazone affected the amount of radioactivity found in these peaks. However, 5-keto clomazone and fosmidomycin inhibited the formation of the product eluting at 8-10 min. In addition, both 5-keto clomazone and fosmidomycin inhibited formation of the product eluting at 13 min. Further studies with 5-keto clomazone showed that inhibition of production of the 13 min peak was dose dependent (Figure 3.3).

No product were formed from from pyruvate in initial experiments using a 330 mM sorbitol concentration, the reaction buffer. In later studies, using

0.165 mM sorbitol, chloroplasts fed pyruvate formed nine ^{14}C -products which partitioned into the aqueous phase (Figure 3.4). 5-keto clomazone prevented production of the compounds eluting at 66.3, 67.9, and 69.9 min (Figure 3.4). No inhibition of these peaks was seen in the 5-OH clomazone or clomazone treatments (data not shown). Fosmidomycin inhibited formation of the products eluting at 66.3 and 69.9 min but not the product eluting at 67.9 min. As fosmidomycin inhibits the second step of chloroplastic isoprenoid biosynthetic pathway, DOXP reductoisomerase (Schwender et al., 1999) and 5-keto clomazone inhibited formation of an additional product than fosmidomycin in the pathway, we hypothesized that 5-keto clomazone may inhibit an earlier enzyme, DOXP synthase, than the step inhibited by fosmidomycin.

Inhibition of heterologous expressed DOXP synthase by 5-keto clomazone

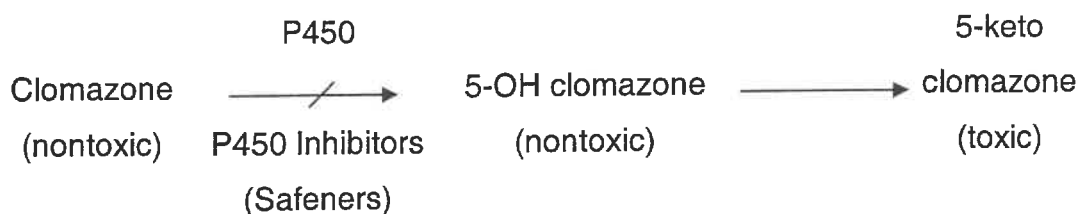
DOXP synthase activity in crude extracts from *E. coli* expressing the DOXP synthase gene from *C. roseus* was inhibited 47% by 20 μM 5-keto clomazone (Table 3.1). The inhibition of DOXP synthase was more severe with the plant DOXP synthase as the bacterial DOXP synthase activity was only inhibited 17% (Table 3.1). Neither clomazone (20 μM) nor 5-OH clomazone (20 μM) inhibited the activity (data not shown).

DISCUSSION

Neither the 'bleaching herbicide' clomazone nor its metabolite, 5-OH clomazone, inhibits the DOXP/MEP pathway in spinach chloroplasts. However 5-keto clomazone, which is proposed to originate from 5-OH clomazone in soybean (ElNaggar et al., 1992) inhibits the DOXP/MEP pathway between pyruvate, GAP and IPP. This was suggested earlier by Zeidler et al. (2000) who showed 5-keto clomazone inhibited emission of isoprene from different

plant species. Our studies with *C. roseus* confirm the results of a previous study with *Chlamydomonas* (Mueller et al., 2000) who showed inhibition of DOXP synthase by 5-keto clomazone. Inhibition of chlorophyll and carotenoid formation by 10^{-5} M clomazone was reversed by 5 mM DOXP (Zeidler et al., 1999).

The clomazone metabolites 5-OH clomazone and 5-keto clomazone were earlier shown to cause bleaching symptoms in plants (Chang et al., 1987). Earlier studies in our laboratory showed that clomazone is a pro-herbicide that is converted to hydroxylated compounds in microsomes by cytochrome P450 (Ferhatoglu et al., 2000). Suicide substrate cytochrome P450 inhibitors, including the organophosphate insecticide phorate, inhibited the hydroxylation reaction. Safening of cotton against clomazone by phorate is due to the inhibition of formation of 5-keto clomazone through inhibition of 5-OH clomazone formation. Our studies also show that neither clomazone (Mueller et al., 2000) nor 5-OH clomazone inhibits carotenoid biosynthesis or DOXP synthase in DOXP/MEP pathway. Only 5-keto clomazone inhibits both. Our results indicate that 5-keto clomazone is the active toxicant causing bleaching in clomazone treated plants. This is illustrated below.



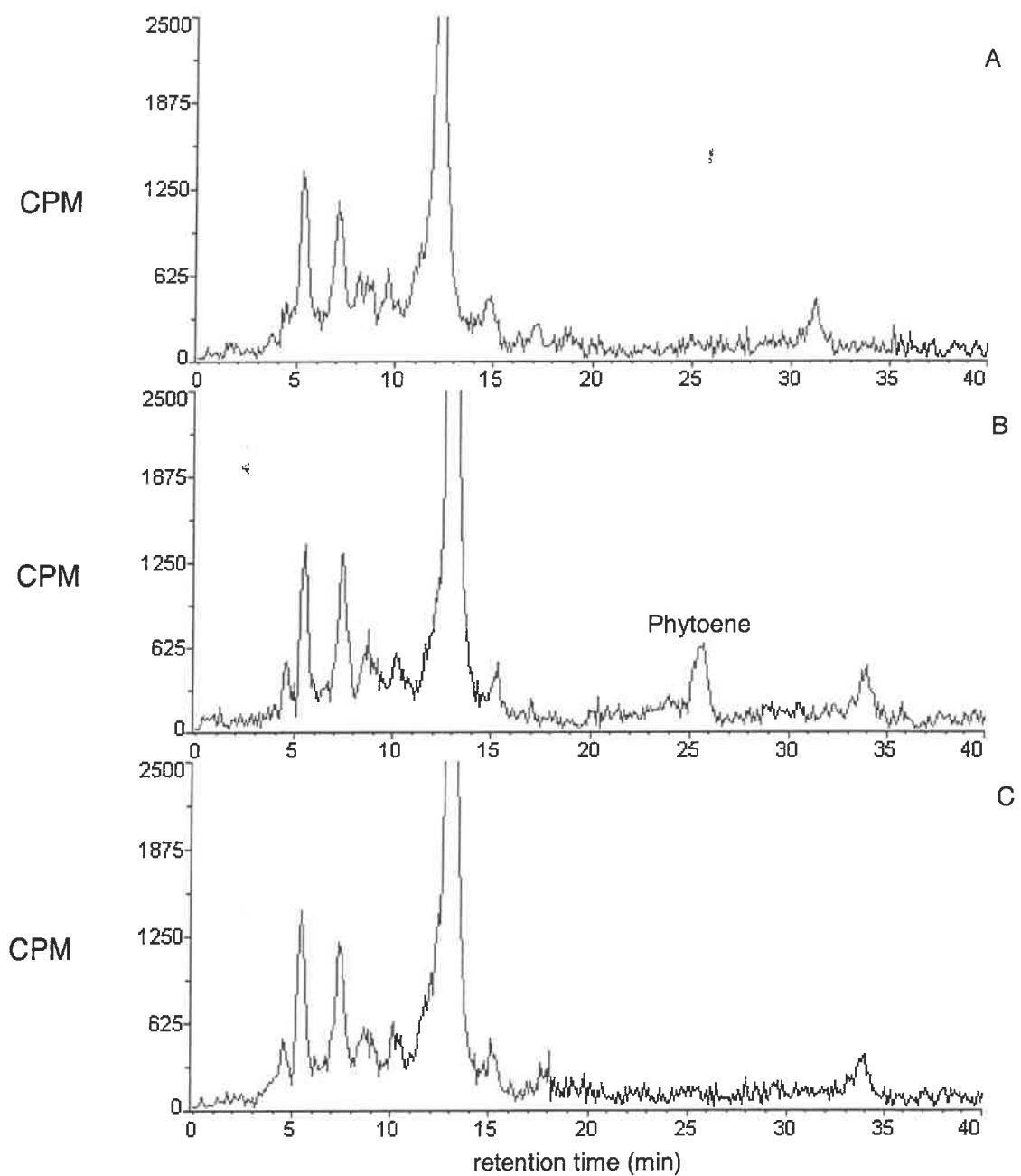
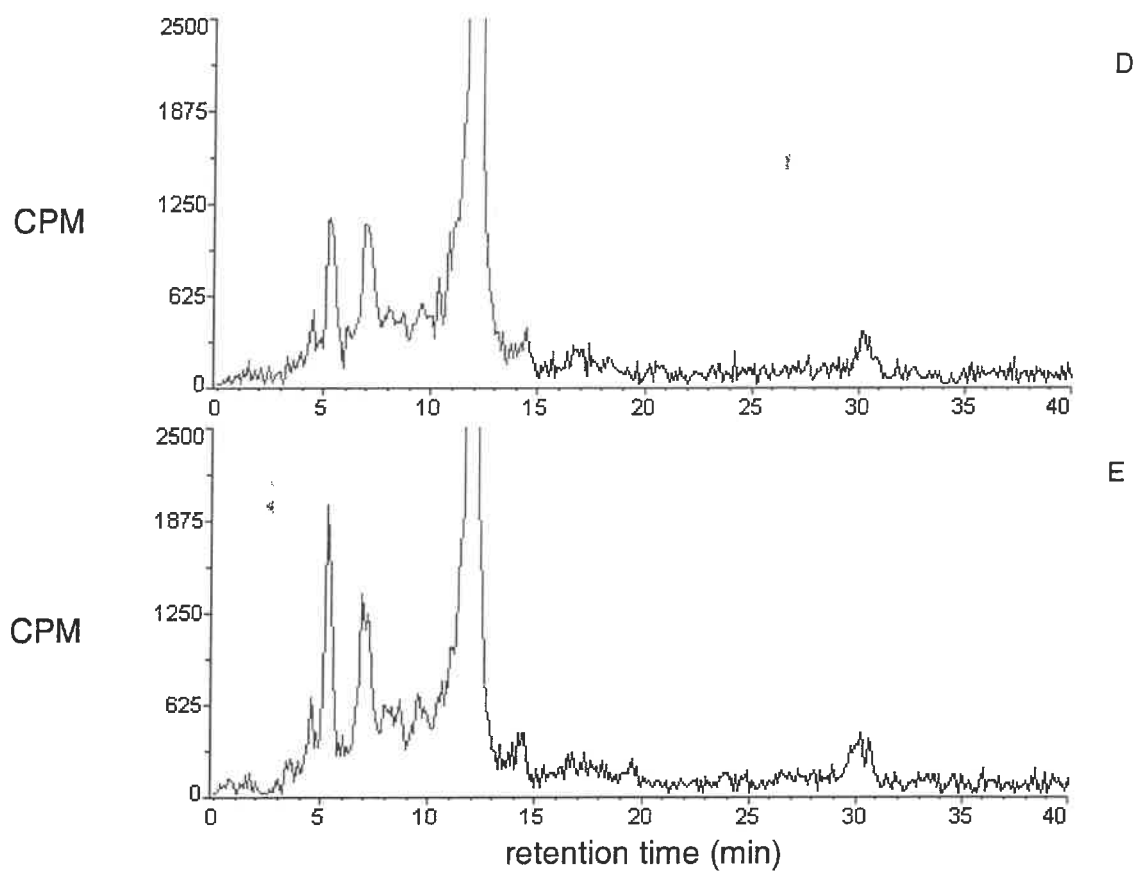


Figure 3.1: Representative HPLC chromatographs of the organic phase from IPP feed spinach chloroplasts treated with norflurazone, 5-OH clomazone, 5-keto clomazone, and clomazone. A. Control; B: Norflurazone; C: 5-OH clomazone; D: 5-Keto clomazone; E: Clomazone.

Figure 3.1 (continued)



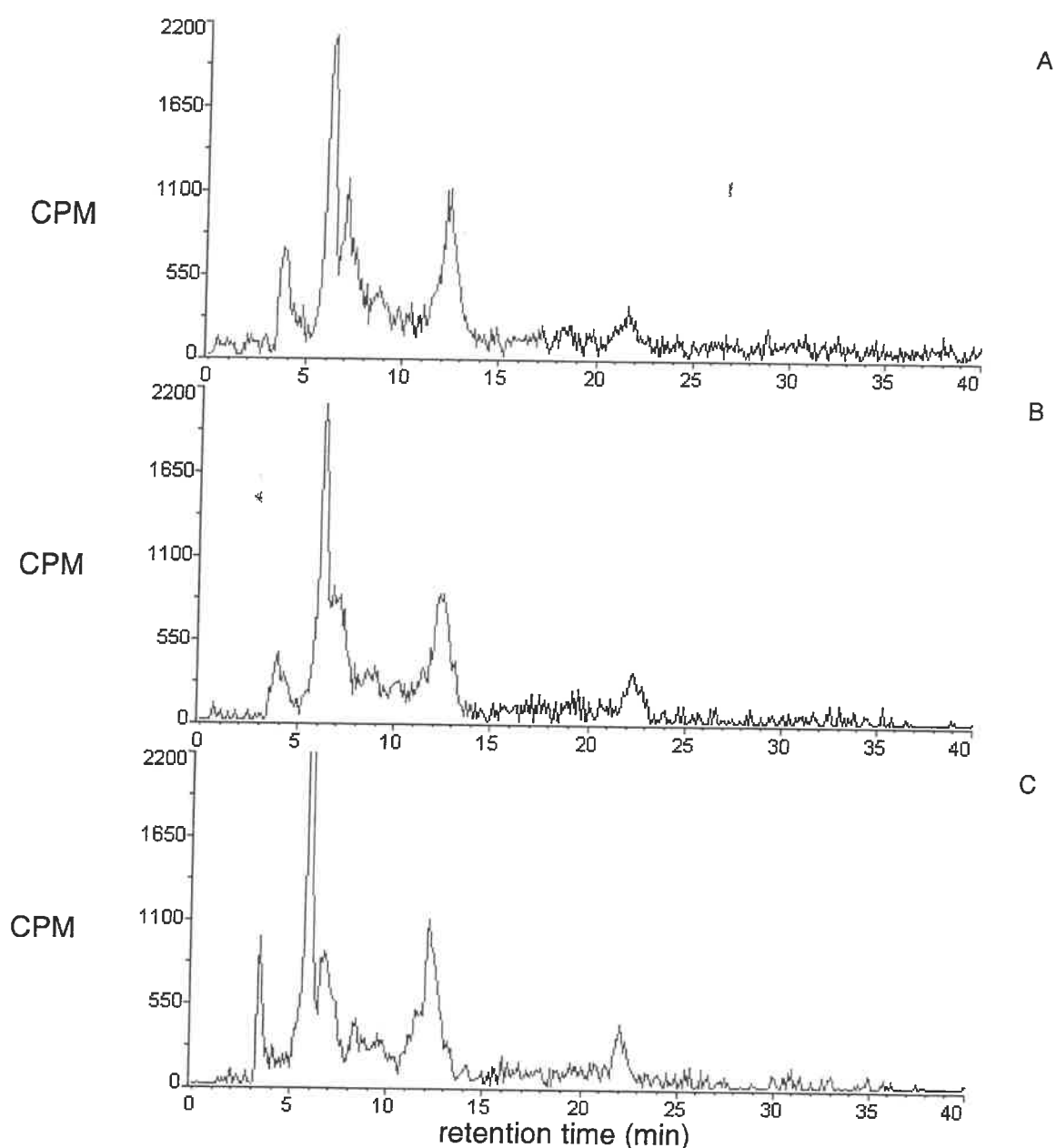
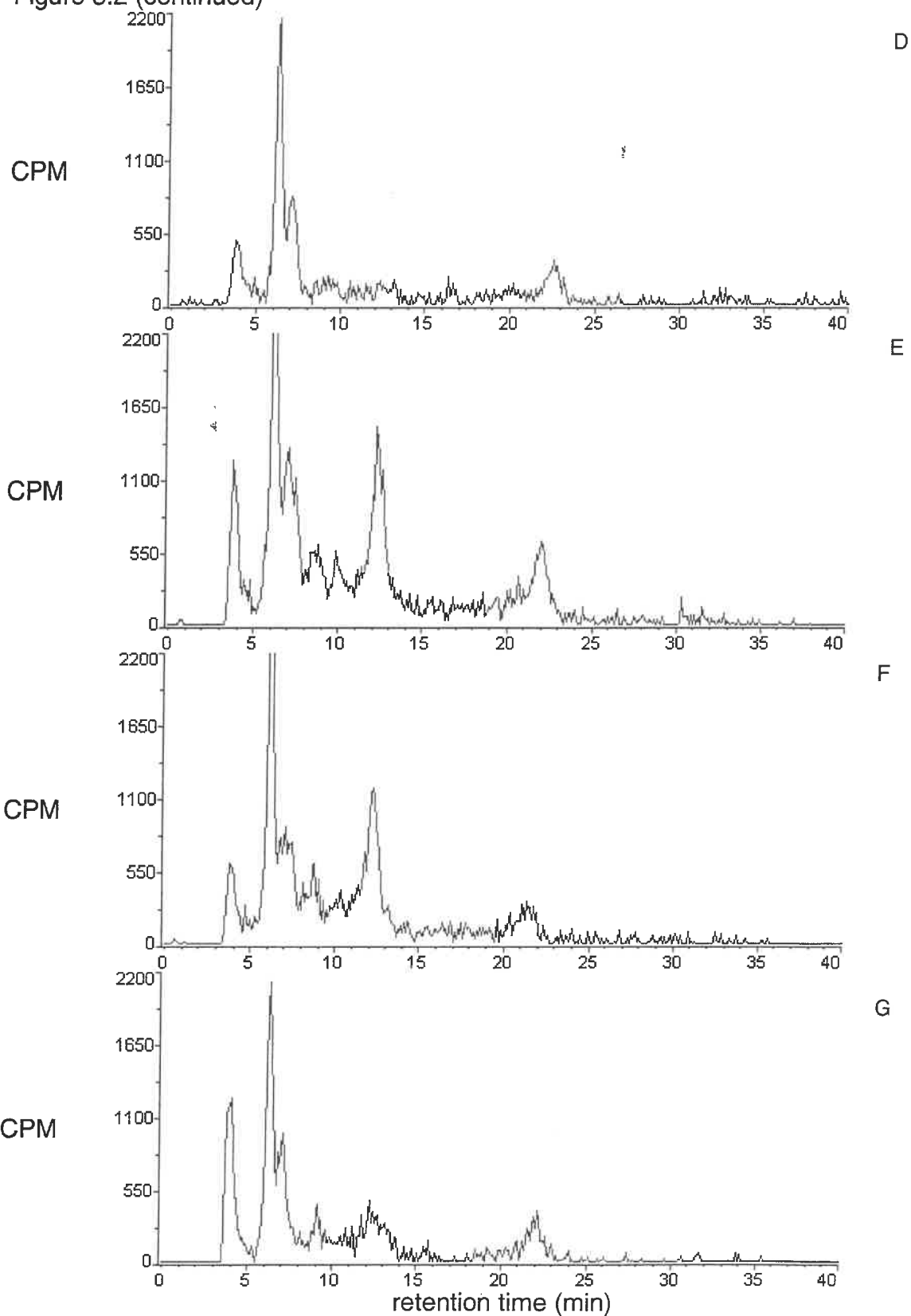


Figure 3.2: Representative HPLC chromatographs of the organic phase from pyruvate feed spinach chloroplasts treated with norflurazone, 5-OH clomazone, 5-keto clomazone, clomazone, and fosmidomycin. A. Control (0.1 % by volume ethanol) for norflurazone, 5-OH clomazone, 5-keto clomazone, and clomazone treatment; B: Norflurazone; C: 5-OH clomazone; D: 5-Keto clomazone; E: Clomazone; F: Control (no ethanol) for fosmidomycin treatment; G: Fosmidomycin.

Figure 3.2 (continued)



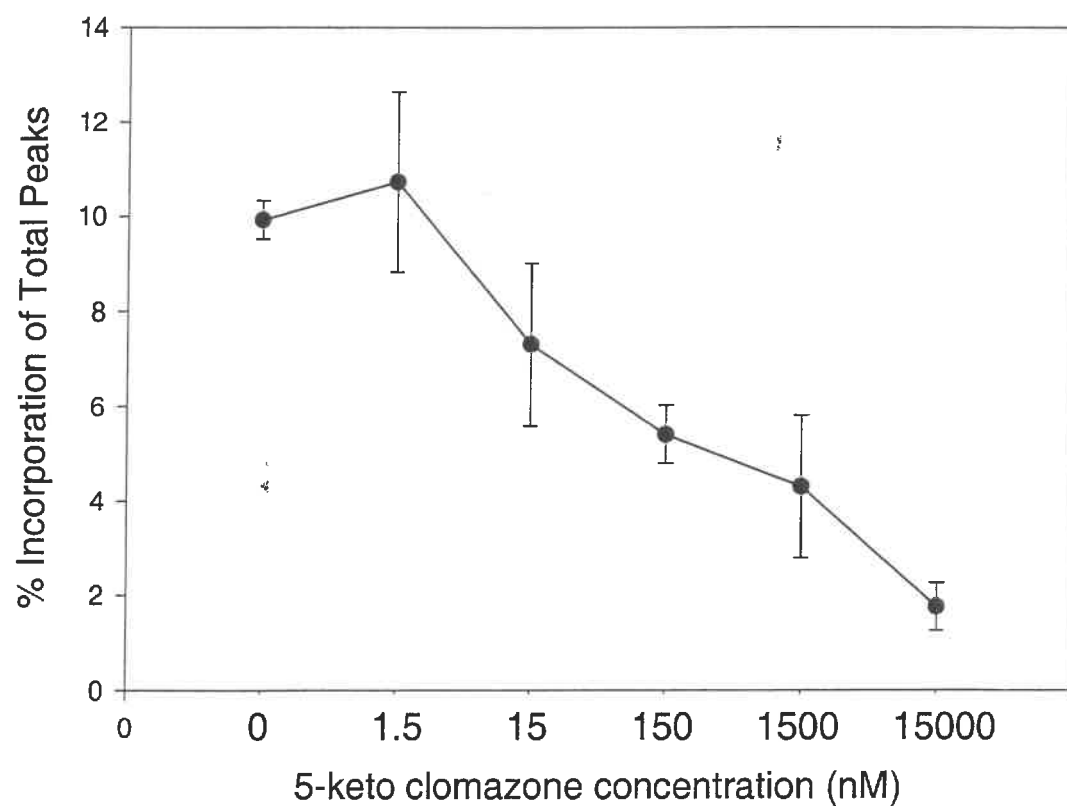


Figure 3.3: Effect of 5-keto clomazone on the radioactivity associated with the peak eluting at 13 min from pyruvate fed spinach chloroplasts. Results are the mean $\pm$ SD.

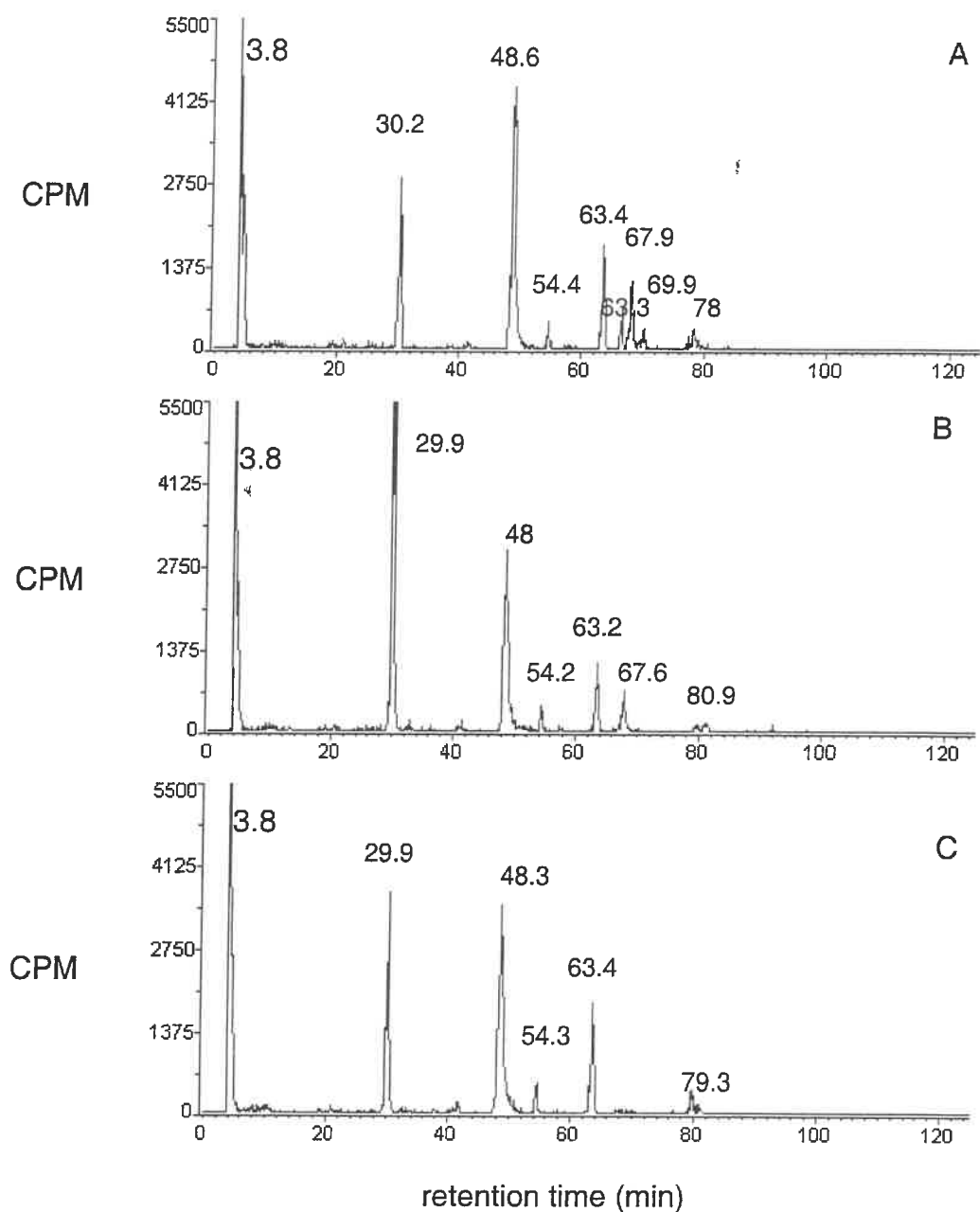


Figure 3.4: HPLC chromatographs of the aqueous phase from ^{14}C -pyruvate feed chloroplasts treated with fosmidomycin and 5-keto clomazone. A. Control; B. Fosmidomycin; C: 5-Keto clomazone.

Table 3.1: Inhibition of *C. roseus* DOXP synthase activity by 5-keto clomazone

DOXP synthase ^a	5-keto treatment	DOXP synthase activity	Inhibition
	(μ M)	(pmol min ⁻¹ mg ⁻¹ protein)	(% of untreated control)
-	0	60 $\pm$ 18	-
-	20	50 $\pm$ 13	17
+	0	1700 $\pm$ 105	-
+	20	900 $\pm$ 53 *	47

^a - =Extracts from *E. coli* cells transformed with vector minus the DOXP synthase gene from *C. roseus*. += Extracts from *E. coli* cells transformed with vector plus the DOXP synthase gene from *C. roseus*

* Indicates a significant difference ($P < 0.05$) compared to the untreated chloroplast within same column.

SUMMARY

Studies were conducted to examine the safening of clomazone on cotton by phorate. Treatment of roots of cotton seedlings grown hydroponically with clomazone reduced both chlorophyll and carotenoid content in a rate dependent manner. Inclusion of phorate in solution partially prevented the loss of chlorophyll and carotenoids also in a rate dependent manner. Further studies showed that it was critical, for maximum safening, that the phorate be present at the same time as the clomazone. Phorate decreased metabolism of clomazone to polar metabolites in excised cotton shoots and shoots of intact cotton plants. Phorate did not affect clomazone metabolism in roots of intact cotton plants. Using HPLC analysis to analyze the polar metabolites, there was no clear reduction in any single polar metabolite. Further studies were conducted to compare the effect of phorate on clomazone metabolism in excised cotton shoots to that of known P450 inhibitors in our lab. Some of the P450 inhibitors tested reduced clomazone metabolism to polar metabolites. This is further evidence for P450 involvement in clomazone metabolism in cotton. In an additional study, metabolism of clomazone in microsomes isolated from corn shoots grown from naphthalic anhydride treated seed was examined. Production of one metabolite, synthesized by P450 and cochromatographing with 5-OH clomazone, was inhibited by phorate. It was suspected that the 5-OH clomazone may be the toxic metabolite that causes bleaching on clomazone treated plants. Synthesis of 5-OH clomazone from clomazone was previously proposed to occur in soybean (ElNaggar et al., 1992). In this scheme, 5-OH clomazone is metabolized further to 5-keto clomazone. 5-OH clomazone, 5-keto clomazone, and clomazone are known to have bleaching activities (Chang et al., 1987). Treatment of cotton seedlings with 5-OH clomazone caused bleaching. Unlike the safening of clomazone by phorate, phorate did not prevent the bleaching following 5-OH clomazone treatment. Approximately 10 times more 5-OH clomazone was needed to

obtain similar bleaching compared to clomazone. These studies show that phorate is inhibiting production of toxic clomazone metabolites.

Studies conducted to test the effect of clomazone and the clomazone metabolites, 5-OH clomazone, and 5-keto clomazone, which we suspected of causing the clomazone toxicity, on the chloroplastic isoprenoid pathway showed no inhibition of the synthesis of products formed from IPP. However, 5-keto clomazone and fosmidomycin inhibited the synthesis of one compound from pyruvate in the organic phase, which cochromatographed with lutein. Analysis of the aqueous phase products from pyruvate feeding showed inhibition of a product eluting at 67.6 min by 5-keto clomazone but not by 5-OH clomazone or clomazone. Since this product was not inhibited by fosmidomycin. It was suspected that 5-keto inhibits an enzyme DOXP synthase, before the fosmidomycin inhibition site studies. Testing a DOXP synthase enzyme from *C. roseus* in-vitro, showed 5-keto clomazone inhibits the enzyme, not 5-OH clomazone or clomazone. This was confirmed recently by Mueller et al. (2000). A scheme of the chloroplastic and plastidic isoprenoid pathway (Lichtenthaler et al., 1999) and mode of action of 5-keto clomazone is shown in Figure 3.5.

Overall, results from safening of the clomazone by phorate and inhibition of DOXP synthase by 5-keto clomazone suggested that phorate inhibits toxic clomazone metabolic pathway by inhibiting synthesis of 5-keto clomazone. Further, 5-keto clomazone is the compound causing bleaching in clomazone treated plants. Although in cotton metabolites, 5-keto clomazone was detected at very low levels in hydrolyzed treatment. A possible explanation might be that synthesis of 5-OH clomazone and then 5-keto clomazone is a rapid reaction. Further studies should be performed to better understand the toxic clomazone pathway in plants.

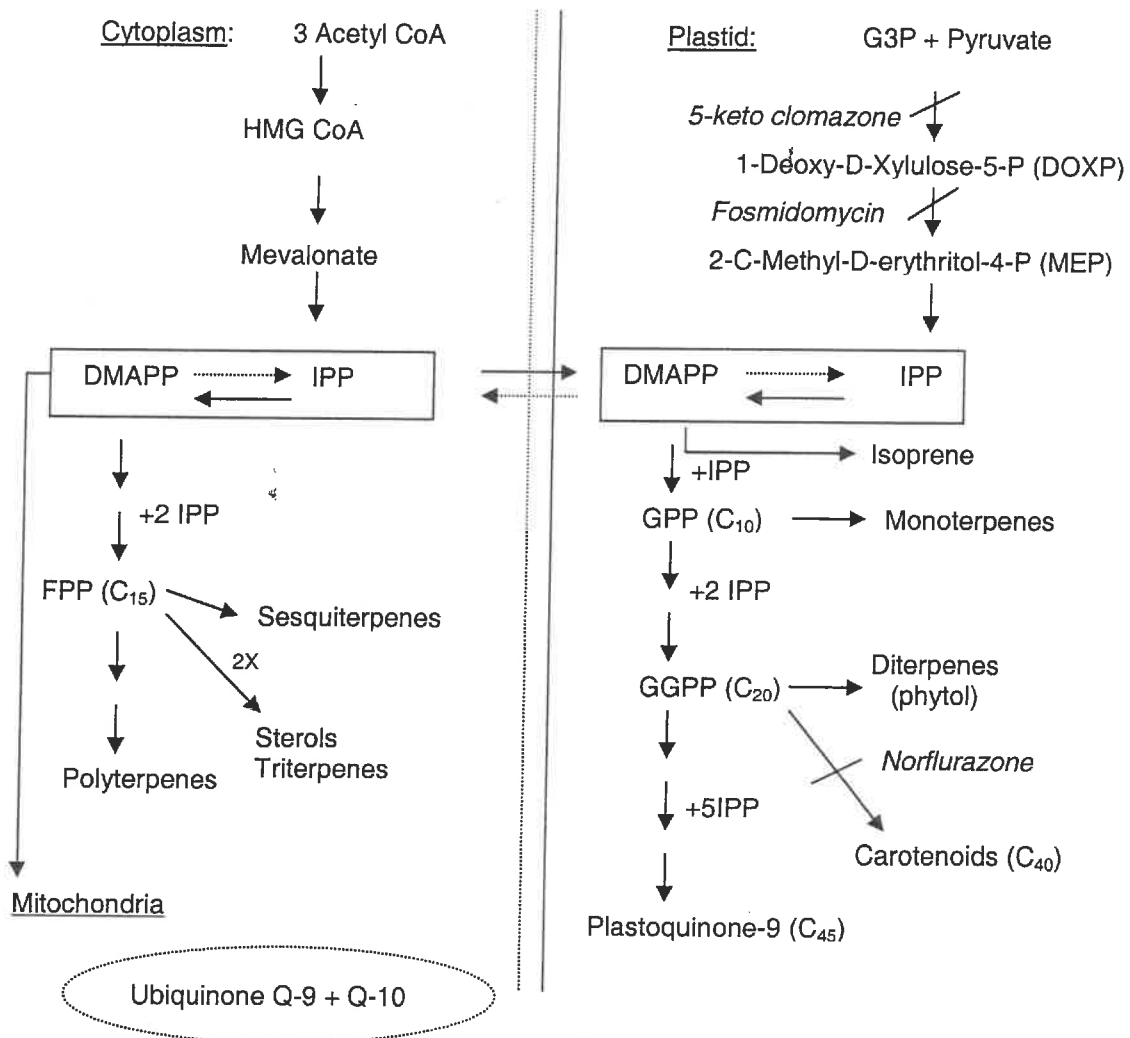


Figure 3.5. Cytoplasmic and plastidic isoprenoid pathway

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