

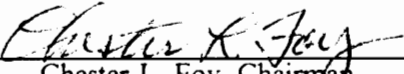
Mechanisms of Action and Selectivity of the Cyclohexen-one  
Herbicide Cycloxydim (BAS 517)

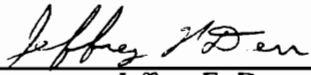
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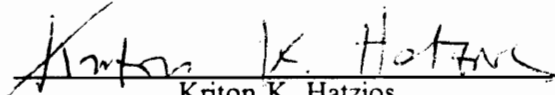
Hwei-Yüing Li

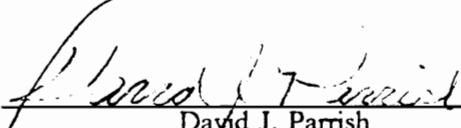
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in partial fulfillment of the requirements for the degree of  
Doctor of Philosophy  
in  
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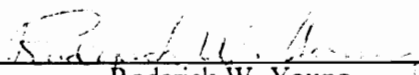
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September 27, 1990

Blacksburg, Virginia

**Mechanisms of Action and Selectivity of the Cyclohexen-one  
Herbicide Cycloxydim (BAS 517)**

by

Hwei-Yüing Li

Chester L. Foy, Chairman

Plant Physiology and Weed Science

(ABSTRACT)

The activity and the selectivity of cycloxydim {2-[1-(ethoxyimino)butyl]-3-hydroxy-5-(2H-tetrahydrothiopyran-3-yl)-2-cyclohexen-1-one}, code designation BAS 517, were examined first with etiolated seedlings of corn (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.]. Etiolated soybean seedlings were not affected by cycloxydim. The degree of growth inhibition of corn varied with concentration of cycloxydim and incubation time. Compared to mesocotyls and coleoptiles, radicles of corn were the most sensitive to cycloxydim. Meristematic tissues appeared to be the site of action of cycloxydim as root meristems were the first to show symptoms. A band of reddening tissue developed at meristematic tips followed by the complete cessation of root growth. In a study comparing activities of technical grade and formulated cycloxydim and sethoxydim, {2-[1-(ethoxyimino)butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one}, formulated compounds were more potent than the technical grade chemicals without formulation additives. Technical sethoxydim was more potent than technical cycloxydim.

Root tips excised from corn and soybean seedlings were used subsequently for cycloxydim treatments. The activity and selectivity of cycloxydim expressed at the isolated root tip level were similar to those of cycloxydim bioassayed with whole seedlings. However, root tips appeared to be more sensitive than the whole seedlings.

Injury at the tissue and cell levels of the 2-mm root tips that were treated with various concentrations of cycloxydim was examined after 24 hours incubation. Concentrations of 0.1, 1, and 10  $\mu$ M cycloxydim caused severe cell vacuolization. A gradient of decreasing injury from epidermal cells toward the center of roots was observed. This pattern of injury appeared to reflect the penetration of cycloxydim into roots along a concentration gradient.

Effects of cycloxydim on  $^{14}\text{C}$ -glucose and -acetate metabolism were examined using excised corn and soybean root tips under tissue culture conditions. The incorporation of  $^{14}\text{C}$  from either precursor into water-soluble compounds and lipids in soybean root tissues was not affected by 10  $\mu\text{M}$  cycloxydim after 4 hours incubation. The incorporation of  $^{14}\text{C}$  from either  $^{14}\text{C}$ -glucose or -acetate into lipids in corn tissues was inhibited by 1  $\mu\text{M}$  cycloxydim after 4 hours incubation. Incorporation of  $^{14}\text{C}$  from  $^{14}\text{C}$ -acetate into lipids was higher than that from  $^{14}\text{C}$ -glucose. Lipids of roots incubated with  $^{14}\text{C}$ -acetate were thus further analyzed. Radioactivity of phosphatidylethanolamine (a fatty acid containing lipids) was reduced in cycloxydim-treated roots; whereas sterols in these roots were not adversely affected. A relatively high degree of variation existed in the  $^{14}\text{C}$  labelling in water-soluble compounds such as total sugars, amino acids, and carboxylic acids in corn tissues. This variability suggests that synthesis of these water-soluble compounds is affected indirectly by cycloxydim.

Acetyl CoA carboxylase (ACCase) is the first committed enzyme involved in fatty acid synthesis. After isolation from root tissues of corn, ACCase was similarly inhibited by cycloxydim and sethoxydim in a dose-dependent fashion. Soybean root ACCase was affected only slightly by 100  $\mu\text{M}$  sethoxydim. The inhibition of ACCase apparently initiates chronic effects observed subsequently in this study, including the inhibition of lipid synthesis and growth and the development of tissue reddening symptoms in roots of corn.

## Acknowledgements

I would like to express sincere appreciation to my major advisor, Dr. C. L. Foy, for his guidance and support during this program. Appreciation is also expressed to Dr. J. F. Derr, Dr. K. K. Hatzios, Dr. D. J. Parrish, and Mr. R. W. Young for serving on my committee. Thanks are due BASF company for providing technical and formulated compounds and partial financial support for this work. Thanks are also due Dr. Robert H. White and Dr. T. W. Keenan in the Department of Biochemistry and Nutrition for their advice and generously loaning laboratory space and equipment for the enzyme work; also Dr. M. Lentner, in the Department of Statistics for assistance in statistical analysis. Special thanks are due David Johnson for his friendship and assistance during the study. Lastly, I express my deepest gratitude to my parents and brother and sisters for their understanding, encouragement, and support.

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## 1.0 INTRODUCTION

Cycloxydim, also known by code designation BAS 517, {2-[1-(ethoxyimino)-butyl]-3-hydroxy-5-(2H-tetrahydrothiopyran-3-yl)-2-cyclohexen-1-one}; is a potent postemergence herbicide recently developed for grass weed control in dicotyledonous crops. Susceptible plants gradually develop injury symptoms a few days following field application of cycloxydim. In susceptible plants, symptoms of tissue reddening and necrosis develop not only on the leaves in contact with the spray but also on other plant parts. This injury pattern reveals the systemic nature of this herbicide. In addition, meristematic regions appear to be the sensitive sites.

In the aspects of field application, weed control spectrum, and symptom development, cycloxydim resembles compounds that are structurally analogous (cyclohexen-ones) and structurally distinct (polycyclic alkanolic acids, abbreviated as PCAs). These compounds were all recently developed for their activity against grass weeds. Cycloxydim resembles sethoxydim {2-[1-(ethoxyimino)butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one} in chemical structure. Sethoxydim was the first cyclohexen-one registered for commercial use in the United States. Cycloxydim and sethoxydim differ in a side group attached to the cyclohexane ring. This side group is a thiopyran ring in cycloxydim, whereas this side group opens up as an ethylthio propyl chain in sethoxydim.

Substantial information relating to sethoxydim and some PCA compounds has been published recently. Despite the chemical differences, these compounds are remarkably similar in their morphological, physiological and biochemical effects. The bases of selectivity of these compounds cannot be explained completely by the differences in absorption, translocation, and metabolism, but can be explained by the different biochemical responses of plants to these herbicides. In the mode of action studies, inhibition of lipid biosynthesis has been demonstrated to be the primary effect of representative compounds of cyclohexen-ones and PCAs; whereas, photosynthesis, respiration, and the syntheses of proteins and nucleic acids were relatively insensitive to these herbicides.

Since leaves are the application target of the new postemergence grass herbicides, leaves, leaf cells, and isolated leaf chloroplasts have often been used as research materials for studying these herbicides. Synthesis of fatty acids in green leaves is strictly localized in chloroplasts. Recently acetyl CoA carboxylase (ACCase), the first committed enzyme involved in fatty acid synthesis, was isolated from leaf tissue and shown to be inhibited by representative compounds of cyclohexen-ones and PCAs. ACCase has also been found in other plant tissues, including developing seeds, embryonic tissues, and cells in tissue culture.

Although root tissues are achlorophyllous and remote from the actual application site, root growth and lipid synthesis in root meristems were reported to be inhibited when root systems were directly treated with sethoxydim and the PCA diclofop  $\{(\pm)\text{-}2\text{-}[4\text{-}(2,4\text{-dichlorophenoxy})\text{-phenoxy]propanoic acid}\}$ . Meristematic root tips are fast growing and metabolically active tissues and are suitable for use in metabolic research. It has not been established whether root tissues can be used representatively for the plant's response to these herbicides.

Research on cycloxydim has demonstrated that it inhibits ACCase isolated only from leaf chloroplasts of susceptible plants. Cycloxydim is structurally different from sethoxydim and therefore it could conceivably have a different or additional mode of action in plants than that of lipid inhibition. In this dissertation, cycloxydim was examined with respect to its biological selectivity and its morphological, physiological, and metabolic effects on susceptible corn plants, with the focus strictly on the root system. The specific objectives of this dissertation were as follows:

1. to determine the selectivity and inhibitory activity of cycloxydim using whole seedlings and the excised plant parts shown to be the most sensitive tissue;
2. to examine the effects of cycloxydim on the morphology of corn (*Zea mays* L.) root tips;
3. to investigate the effects of cycloxydim on <sup>14</sup>C-glucose and -acetate metabolism, which includes glycolysis, TCA cycle function, and lipid synthesis in roots; and
4. to isolate and examine the ACCase from root systems of tolerant soybean [*Glycine max* (L.) Merr.] and susceptible corn plants in response to cycloxydim treatments.



## 2.0 LITERATURE REVIEW

### 2.1 INTRODUCTION

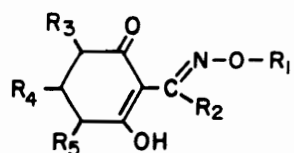
Cyclohexen-ones and polycyclic alkanolic acids (PCAs or aryloxyphenoxypropionic acids) are two groups of postemergence herbicides recently developed to control annual and perennial grasses in dicotyledonous crops. Low dosage rates are required making these herbicides superior in many aspects to herbicides developed for grass weed control in the 1950s and 1960s (e.g. chloroacetamides and thiocarbamates). Most of the earlier herbicides were preemergence-applied or soil-incorporated. The modern herbicides are used conveniently as postemergence sprays after a grass weed problem has developed. They are systemic and more potent than the older grass herbicides.

In addition, the cyclohexen-ones and PCAs have low residual activity and relatively low mammalian toxicity. Due to increasing concerns of public health and environmental pollution, these characteristics are common to most of the herbicides developed recently. The modes of action of the thiocarbamates and chloroacetamides are still unclear, while that of PCAs and cyclohexen-ones are better known at the enzyme level. Based on these superior characteristics and the continuing research to improve formulations, weed control programs, and gene-engineered herbicide-resistant crops, an increase in the use of these modern herbicides is highly likely.

The herbicide (cycloxydim) studied in this dissertation is a member of the cyclohexen-ones; but when preparing this literature review, a consideration of the PCAs also seemed appropriate. Recently, the mode of action of these two different groups of herbicides was demonstrated to be the same. The research on PCAs contributed much toward revealing the mode of action of these modern herbicides. Reviews on the development, activity, and mode of action of PCAs have been published recently (22, 74). This review will only compare the two groups of herbicides generally, highlighting the similarities and differences.

## ***2.2 CHEMICAL DIFFERENCES BETWEEN CYCLOHEXEN-ONE AND PCA HERBICIDES***

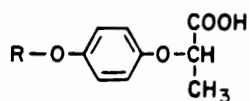
Cyclohexen-ones, as the name implies, contain a common cyclohexane ring with substituted groups attached to it. PCAs are composed of an alkanic acid with a group containing more than one ring structures attached to an asymmetric carbon of the alkanic acid. According to Duke (22), the PCAs can be further divided into the oxyphenoxy alkanic acids (generally termed the "fops") and the benzoyl-*N*-phenyl phenoxy propanoic acids (often termed "props"). The structures of representative examples of cyclohexen-ones and PCAs are shown in Figures 2-1 and 2-2, respectively. Tables 2-1 and 2-2 show the common names, trade names, and names of the manufacturers of the representative chemicals.



Herbicide	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>
Sethoxydim	C <sub>2</sub> H <sub>5</sub>	C <sub>3</sub> H <sub>7</sub>	H <sub>2</sub>		H <sub>2</sub>
Clethodim	CH <sub>2</sub> CH=CHCl	C <sub>2</sub> H <sub>5</sub>	H <sub>2</sub>		H <sub>2</sub>
Cycloxydim	C <sub>2</sub> H <sub>5</sub>	C <sub>3</sub> H <sub>7</sub>	H <sub>2</sub>		H <sub>2</sub>
Trelkoxydim	C <sub>2</sub> H <sub>5</sub>	C <sub>2</sub> H <sub>5</sub>	H <sub>2</sub>		H <sub>2</sub>
Alloxydim	CH <sub>2</sub> CH=CH <sub>2</sub>	C <sub>2</sub> H <sub>5</sub>	COOCH <sub>3</sub>	H <sub>2</sub>	

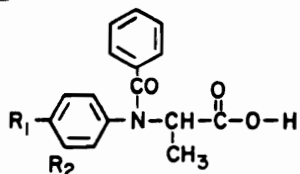
Figure 2-1. Structures of cyclohexen-one herbicides.

### OXYPHENOXY



Herbicide	R
Fluazifop	
Haloxifop	
Diclofop	
Fenoxaprop	
Quizalofop	
Fenthiaprop	

### BENZOYL-N-PHENYLS



Herbicide	R <sub>1</sub>	R <sub>2</sub>
Benzoylprop	Cl	Cl
Flamprop	Cl	F

Figure 2-2. Structures of polycyclic alkanolic acid herbicides.

Table 2-1. Cyclohexen-one herbicide names and manufacturers.

Common name†	Trade name	Chemical name	Manufacturer
Sethoxydim■	Poast	2-[1-(ethoxyimino)-butyl]-5-[2-(ethylthio)-propyl]-3-hydroxy-2-cyclohexen-1-one	BASF
Clethodim■	Select	(E,E)-2-[1-[[3-chloro-2-propenyl)oxy]imino]propyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one	Valent
Cycloxydim	Laser Focus Stratos	2-[1-(ethoxyimino)-butyl]-3-hydroxy-5-(2H-tetrahydrothiopyran-3-yl)-2-cyclohexen-1-one	BASF
Tralkoxydim	Grasp	2-[1-(ethoxyimino)-propyl]-3-hydroxy-5-mesitylcyclohex-2-enone	ICI
Alloxydim-sodium	Clout	2-(1-allyl-oxyimino-butylidene)-5,5-dimethyl-4-methoxycarbonylcyclohexane-1,3,-dione	Nippon Schering

† Chemicals followed by squares are registered in the United state. The chemical without a square is registered elsewhere.

**Table 2-2. Polycyclic alkanolic acid herbicide names and manufacturers.**

Common name†	Trade name	Chemical name	Manufacturer
Fluazifop■	Fusilade	(±)-2-[4-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic acid	ICI
Haloxifop■	Verdict	2-[4-[[3-chloro-5-trifluoromethyl-2-pyridinyl]oxy]phenoxy]propanoic acid	Dow
Diclofop■	Hoelon	(±)-2-[4-(2,4-dichlorophenoxy)phenoxy]propanoic acid	Hoechst Roussel
Fenoxaprop■	Whip, Acclaim	(±)-2-[4-[(6-chloro-2-benzaxazolyl)oxy]phenoxy]propanoic acid	Hoechst
Quizalofop■	Assure	(±)-2-[4[(6-chloro-2-quinoxalinyloxy]phenoxy]propanoic acid	DuPont

† Haloxifop and diclofop are formulated as methyl esters  
 Fenoxaprop and quizalofop are formulated as ethyl esters.  
 Fluazifop is formulated as butyl ester.  
 Chemicals followed by squares are registered in the United state.

Cyclohexen-ones and PCAs are also different in regard to light sensitivity and formation of isomers during synthesis. Rapid transformation of sethoxydim under light was reported by Campbell and Penner (11). At least six radiolabeled compounds were found after a 1-hour exposure of radiolabeled sethoxydim to light. Two of these compounds were demonstrated to be phytotoxic. The ethoxy alkyl side chain of sethoxydim appeared to be an essential component for the mode of action. Once this group was lost, sethoxydim became nonphytotoxic.

Due to the presence of a chiral carbon (an asymmetric carbon) in the PCAs' structures, R and S isomers or enantiomers are formed during chemical synthesis (22, 25). R enantiomers are biologically active, and S enantiomers are relatively inert. In addition, acids are the active forms of PCAs. In order to facilitate absorption into plant foliage, all PCAs are commercially made as esters. The esters are hydrolyzed in the plants to the parent acids for activation.

## ***2.3 SIMILARITIES AND DIFFERENCES OF CYCLOHEXEN-ONES AND POLYCYCLIC ALKANOIC ACIDS (PCAS) IN RELATION TO HERBICIDAL ACTIVITY***

### **2.3.1 Weed Control Spectra**

The spectra of weeds controlled by cyclohexen-ones and PCAs are very similar. Generally speaking, dicotyledonous species are tolerant and many monocotyledonous species are susceptible to both groups of herbicides. In monocotyledoneae, the grass-like Liliaceae species are tolerant to these two groups of herbicides. Most of the agronomic monocotyledonous weeds are species of

Poaceae and Cyperaceae, which are in the order Graminales. These two groups of herbicides have been called "graminicides". However the herbicides do not control all plants in Graminales as the name might imply. The sedges (Cyperaceae) are tolerant to both groups of herbicides.

Most members of Poaceae are susceptible to these two groups of herbicides. Species reported as tolerant to either sethoxydim or fluazifop include red fescue (*Festuca rubra* L. # FESRU), tall fescue (*Festuca arundinacea* Schreb. 'Ky 31' # FESAR), hard fescue (*Festuca longifolia* Thuill), rattail fescue [*Vulpia myuros* (L.) K. C. Gmel. # VLPMY], annual bluegrass (*Poa annua* L.), and centipedegrass (*Eremochloa ophiuroides* (Munro) Hack. 'Common' # ERLOP) (7, 42, 47, 75). Plants show varying responses to cyclohexen-ones and PCA compounds, depending on species (21) or cultivar (7), growth stage (16), environmental condition (16, 23, 27, 47, 59), herbicide formulation (4, 16, 55), and application rate (5, 45). Some PCAs including benzoyl-*N*-phenyl propanoic acid, flumetypyr-methyl, and diclofop-methyl are unique in their safe use in cereal crops such as barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), and rye (*Secale cereale* L.) for wild oat (*Avena fatua* L. # AVEFA) control (22). None of the cyclohexen-one is safe to use on any of the cereal crops.

### 2.3.2 Herbicide Absorption and Translocation

Following foliar application, tolerant and susceptible species absorb cyclohexen-one or PCA herbicides readily (6, 12, 13, 15, 20, 22, 30, 31, 48, 56, 59, 60, 69, 71, 73, 76). Translocation occurs both acropetally and basipetally in all plants examined. Herbicides accumulate in rapidly growing tissues. The patterns of absorption and translocation of both kinds of herbicides are very similar in tolerant and susceptible species. Various factors related to the plant growth stage, environment, and spray formulations influence the absorption and translocation of these herbicides (6, 15, 31, 48, 50, 56, 59, 60, 61, 76, 77).



## 2.3.3 Herbicide Metabolism

### 2.3.3.1 Metabolism of Cyclohexen-ones in Plants

The light-sensitive nature of cyclohexen-ones complicates the studies on metabolism of cyclohexen-ones in plants. Seven out of the nine metabolites of sethoxydim found in treated plants have the same chromatographic characteristics as those found in the light degradation products (12). The metabolism of sethoxydim in the monocotyledonous plants quackgrass [*Agropyron repens* (L.) Beauv. # AGRRE] and barnyardgrass [*Echinochloa crus-galli* (L.) Beauv. # ECHCG], and that of the dicotyledonous plants alfalfa (*Medicago sativa* L. 'Saranac') and navy bean (*Phaseolus vulgaris* L. 'Seafarer'), were reported to be similar, qualitatively and quantitatively. According to other reports (71, 73) differential metabolism of either alloxydim-sodium or sethoxydim was suggested as the basis of selectivity in plants. Greater proportions of unchanged sethoxydim were extracted from the apical leaves and roots of the intact plants and cell cultures of johnsongrass [*Sorghum halepense* (L.) Pers.], the susceptible species, than from soybean [*Glycine max* (L.) Merr.], a tolerant species (71). Three <sup>14</sup>C-metabolites of sethoxydim were found, one the same as the light-degradation metabolite of sethoxydim; however, the structures of these compounds in this study were not identified.

Alloxydim-sodium was readily degraded in plants, and the de-allyloxyated alloxydim was identified as the major metabolite (73). Greater proportions of unaltered <sup>14</sup>C-alloxydim-sodium were found in wild oat, a susceptible species, than in sugarbeet (*Beta vulgaris* L.), a tolerant species. Since all plants absorbed and translocated the herbicides similarly, the authors (71, 73) suggested that soybean and sugarbeet might gain their tolerance by metabolizing the herbicides at a higher rate than the susceptible species.

The metabolism of cycloxydim has been investigated only in soybeans, a tolerant species. There is no information available on the metabolism of cycloxydim in any susceptible species, nor is there any information on the toxicity of the transformed products of cycloxydim in the tolerant

species. Huber et al. (43) reported recently an extensive study on cycloxydim metabolism in soybeans. Plants were treated with  $^{14}\text{C}$ -cycloxydim at the two-leaf stage and then harvested shortly after spraying and at 7, 14, 21, 40, and 82 days post treatment. They reported that cycloxydim was rapidly metabolized in soybeans, and the transformation of this compound was very complex.

The sulfur atom in cycloxydim is readily oxidized on and in the soybean plant to form sulfoxide and sulfone metabolites (43). Therefore only trace amounts of cycloxydim were detected shortly (within one day) after spraying the plants. The sulfoxide and sulfone metabolites and their hydroxylated derivatives were the predominant oxidation products of cycloxydim in soybeans. Some of the metabolites existed in either free or conjugated forms with glucose (43).

The oxidized metabolites could be further metabolized through the oxidative cleavage of the cyclohexenone ring and the cleavage of the ethoxyimino group. Beckmann rearrangement (43) was also responsible for the transformation of the oxidized metabolites, except for their hydroxylated derivatives. Although enzymes may be involved in the cleavage of the ethoxyimino group in plants, this reaction could also be initiated photochemically. The deoxyimino sulfoxide and sulfone metabolites of cycloxydim were formed in abundance when irradiating an aqueous solution of cycloxydim with a xenon arc source simulating sunlight (43).

The transformation products of cycloxydim were different qualitatively and quantitatively depending on the plant part (foliage or seed) investigated. These metabolites of cycloxydim could be further metabolized in soybeans and were found partly incorporated into lignin fractions in foliage and proteins in seeds.

### **2.3.3.2 *Metabolism of PCAs in Plants***

PCA herbicides enter plants as esters. The esters are then hydrolyzed to the active parent acids by carboxylesterase. Some PCAs were reported to be metabolized differently in tolerant and susceptible species (6, 22). Reactions, summarized by Duke (22), occurring in the metabolism of PCAs were the formation of glucose conjugates in susceptible species and aryl hydroxylation and

phenolic conjugation in resistant species. Some metabolic intermediates were also herbicidally active. Difenopenten-ethyl {ethyl 4-[4-(4'-trifluoromethylphenoxy)-phenoxy]-2-pentenoate}, a PCA, was metabolized similarly in tolerant and susceptible species (66).

Overall, the differences in metabolism of PCAs and cyclohexen-ones do not entirely explain the differential sensitivity exhibited by tolerant and susceptible species to these herbicides.

### 2.3.4 Symptoms

The symptoms produced by cyclohexen-one and PCA herbicides are very similar. At the whole plant level, growth inhibition, foliar reddening, and subsequent necrosis generally occur within one to three weeks. Meristematic regions such as apices, intercalary nodes, and rhizome nodes are the first affected (1, 2, 18, 35, 38, 46).

The cessation of growth is related to the cytological number of mitotic cells, and the occurrence of binucleate cells has been observed (3, 39, 40, 51). Cytokinesis is suggested to be disturbed more than was karyokinesis by the herbicides (3, 39, 40). At the cell and tissue levels, injuries observed at meristematic regions of shoots and root tissues include vacuolated cells, aggregated cytoplasm, and disappearance of procambium (39, 72). Ultrastructural damages to chloroplasts include the disruption of the outer chloroplast envelope and a disorganization of the internal thylakoid system in leaves of *Elymus repens* treated with fluzifop (14).

Injuries at cell and tissue levels develop within 1 day in young seedlings. Damages occurring in such generalized patterns do not lead to any specific clue as to the mode of action of these herbicides. Considering the time required to show the effects of herbicide application and the vague information obtained from the morphological studies, the biochemical approaches discussed in the next section were more effective for detecting the mode of action.

### 2.3.5 Physiological and Biochemical Effects

Two physiological effects, membrane disruption and anti-auxin activity are known for the PCAs (19, 65, 67, 78). Investigations related to such effects have not been reported on cyclohexen-ones. Although substantial research data support these two effects of PCAs, the concentrations used are relatively higher than those used to inhibit lipid synthesis. Therefore these effects are considered to be secondary.

At the whole-plant level, susceptible plants treated with some representative cyclohexen-ones and PCAs showed a cessation of growth; reductions of fresh and dry weights, respiratory activity, and photosynthesis activity; and an increase in anthocyanin and total sugar content (3, 15, 29). Lipid biosynthesis was found to be more sensitive to sethoxydim than protein and nucleic acid synthesis and photosynthesis by supplying various  $^{14}\text{C}$ -labeled metabolic precursors to isolated leaf cells of soybean (a tolerant species) (34). Similar results were obtained from experiments using the same approach in leaf tissues and root tissues of corn (*Zea mays* L.) (a susceptible species) treated with sethoxydim and haloxyfop (10, 17, 35, 36, 37, 38, 40, 41, 44, 52). Inhibition of incorporation of  $^{14}\text{C}$ -acetate into polar lipids in root and leaf tissues by these two groups of herbicides could be detected within 0.5 hour after treatment with herbicide concentrations as low as  $1\ \mu\text{M}$ .

Recently, acetyl CoA carboxylase (ACCCase), the first committed enzyme involved in fatty acid synthesis, was found to be inhibited by cyclohexen-ones and PCAs in a dose-dependent fashion (9, 10, 24, 54, 57, 58, 63, 64, 68). Two dehydrogenases (DH),  $\alpha$ -ketoglutarate DH and pyruvate DH, involved in glycolysis and the TCA cycle were reported to be inhibited by haloxyfop (18). Photosynthetic electron transport system inhibition in isolated leaf chloroplasts by quizalofop was reported (62). However, the concentrations reported to inhibit the enzymes were relatively higher than those inhibiting lipid synthesis. The authors stated that the inhibitory effects on these enzymes were possible under the condition that haloxyfop was concentrated in compartments.

Other evidence supporting the inhibition of ACCCase as the primary effect of these two groups of herbicides includes:

1. Inhibition of fatty acid synthesis in isolated grass chloroplasts by representative cyclohexenones and PCAs was reported (52, 54). Lipids that contain fatty acids, such as polar lipids and triglycerides, were reduced in tissues treated with such herbicides, whereas synthesis of sterols was not affected (8, 36, 41). Sterols do not contain fatty acids (53). Sterols are synthesized using the five-carbon isoprene as the building block. Syntheses of fatty acids and sterols proceed by two different pathways (53, 70). ACCase is involved only in the synthesis of fatty acids.
2. ACCase catalyzes the formation of malonyl CoA from acetyl CoA and bicarbonate. Some cyclohexenone and PCA herbicides were reported to inhibit the incorporation of  $^{14}\text{C}$ -acetate and -acetyl CoA into fatty acids using enzyme preparations isolated from chloroplasts (24, 50). The incorporation of  $^{14}\text{C}$ -malonate and  $^{14}\text{C}$ -malonyl CoA into fatty acids was not affected.
3. Clofop-isobutyl [isobutyl 2-[4-(4-chlorophenoxy)-phenoxy]-propionate] is structurally analogous to diclofop-methyl and is an active hypolipidemic drug used in animals to reduce serum cholesterol and triglycerides (28).
4. ACCases isolated from tolerant and susceptible plants show different affinity for cyclohexenones and PCA compounds.  $I_{50}$  values of these herbicides, the concentrations that inhibited ACCase activity by 50%, for ACCases isolated from tolerant species were much higher (0.1 to 0.5 mM) than those (0.4 to 1.2  $\mu\text{M}$ ) from the susceptible species (64).

ACCase has been isolated and studied in animal tissues, microorganisms, and higher plants, both from green and etiolated tissues (26, 49, 68, 70). ACCase is a complex enzyme with dual activities, acting as an acetyl CoA carboxylase and as a carboxyltransferase (26, 32, 33). ACCase is a biotin-containing enzyme. Biotin and its associated component, functioning as an intermediate carrier of the carboxyl group, is able to rotate from one active site to another to carry out the two enzymatic activities.

Data from a detailed kinetic study indicate that the carboxyltransferase site rather than the biotin carboxylation site of the enzyme is inhibited by representative compounds in both herbicide

classes (58). Although these herbicides are of two distinct chemical classes, they might bind to the same region of the enzyme.

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# **3.0 EFFECT OF CYCLOXYDIM ON GROWTH OF CORN AND SOYBEAN SEEDLINGS**

## ***3.1 INTRODUCTION***

Cycloxydim is a potent postemergence herbicide that is currently under development. Similar to other structurally related cyclohexen-ones and structurally distinct PCAs, cycloxydim is active against many grass weeds and therefore is being developed for use in dicotyledonous crops. Prior to the investigation of physiological effects and modes of action of these herbicides, identification of sensitive plant tissues and determination of a range of physiological concentrations are essential. The determination of the activity of cycloxydim is particularly necessary with the knowledge that cycloxydim is light sensitive (12).

Activities of some postemergence herbicides have been studied by monitoring plant growth following foliar application with different dosages in the field (1, 2), greenhouse (3, 6, 16), and growth chamber (6, 9). Measurements of either shoot or root growth of seedlings in response to

treatment of compounds with similar herbicidal activity to that of cycloxydim in the laboratory have been also reported. Radicles of these seedlings were treated with different concentrations of herbicides in plastic bags using herbicide saturated germination papers (7, 18), in petri-dishes using herbicide saturated filter paper discs (6) or silica sand (14), and in hydroponic culture (10). Root systems, although not a direct application target of these herbicides, consistently showed high sensitivity to some members of the two groups of grass herbicides. Determination of activities of some cycloxydim-related herbicides have also been conducted using tissue culture techniques (11).

Growth of root tips excised from seedlings (11) and the viability of cells in suspension culture (5) have been monitored in response to different concentrations of sethoxydim and haloxyfop respectively. These plant materials responded similarly to whole plants treated under field conditions.

In this research, a rag-doll technique was used for cycloxydim bioassay because of its simplicity and economy. Clean seedlings can be quickly and easily obtained by germination under aseptic laboratory conditions. Uniformly grown seedlings can then be selected for herbicide treatment. Intact seedlings, mimicking whole plants exposed in field applications, were sandwiched in germination papers wetted with various concentrations of cycloxydim for treatment. To avoid the inhibitory effects of light on coleoptile elongation of corn seedlings and degradation of cycloxydim under light, etiolated seedlings were treated and the bioassay was conducted in the dark.

The objectives of this research were:

1. to evaluate the feasibility of using this simple, quick, and economical rag-doll procedure for laboratory bioassay of cycloxydim;
2. to examine and quantify the responses of different seedling parts to cycloxydim treatments; and
3. to compare the activities of both formulated and technical grade cycloxydim with those of sethoxydim.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Plant Materials**

#### ***3.2.1.1 Seed Imbibition and Surface Sterilization***

Corn and soybean seeds were soaked in distilled water for approximately 16 to 20 hours until seeds were fully imbibed. Seeds were surface-sterilized in a 33% commercial bleach solution (1.3% sodium hypochlorite) for 15 minutes. Seeds were then transferred to a laminar flow hood, where all procedures were done aseptically. Seeds were rinsed with sterile distilled water until the bleach odor was no longer detectable.

#### ***3.2.1.2 Seed Germination***

Seeds were placed on sheets of germination paper, 25 cm × 37.5 cm (Anchor Paper Co., Saint Paul, Minnesota 55165), moistened with sterile distilled water. The radicles were oriented toward the bottom of the paper and the shoot ends toward the top. Germination papers were rolled up and placed upright in beakers containing a 2.5-cm depth of sterile distilled water. These were placed in pans (previously autoclaved), covered with aluminum foil, and placed in an incubator for germination at 25°C.

### 3.2.2 Preparation of Herbicides for Treatments

Four herbicides used in this experiment were supplied by BASF. The technical cycloxydim contained 65.7% active ingredient (ai). Formulated cycloxydim contained 18% ai (178.8 g/l). Technical grade sethoxydim (BAS 562 20 H) was a lithium salt of sethoxydim with 75% ai, very soluble in water. Poast, a commercial formulation of sethoxydim, contained 18% ai. Sethoxydim was used as a reference standard, since much information on this compound has been published. For treatments, 200 ml of 0.1, 1, 10, and 100  $\mu\text{M}$  of each chemical were prepared .

#### 3.2.2.1 Preparation of Formulated Cycloxydim

A volume of 36  $\mu\text{l}$  formulated cycloxydim ( $36\mu\text{l} \times 178.8 \text{ g/l} \div 325 \text{ (molecular mass)} = 20 \mu\text{mole}$ ) was mixed with distilled water to make 200 ml of a 100  $\mu\text{M}$  solution. From this solution, 10-fold dilutions were made successively in order to prepare 100 ml each of 10, 1, and 0.1  $\mu\text{M}$  concentrations.

#### 3.2.2.2 Preparation of Technical Grade Cycloxydim

Technical cycloxydim (50 mg), which contains approximately 0.1 mmole ( $= 50 \text{ mg} \times 65.7\% \div 327.5 \text{ (molecular mass)}$ ), was placed in a microcentrifuge tube (Brinkmann Instruments Co., Westbury, New York 11590). Acetone (0.5 ml) was added to this tube to dissolve the gummy technical cycloxydim. From this acetone solution, 100  $\mu\text{l}$  containing 20  $\mu\text{mole}$  of cycloxydim were added to 200 ml of distilled water to make a 100  $\mu\text{M}$  cycloxydim technical solution with 0.05% acetone. This solution was diluted successively by 10-fold to make 10, 1, and 0.1  $\mu\text{M}$  solutions. The controls containing 0.05% acetone were not inhibitory to seedling growth.

### **3.2.2.3 Preparation of Formulated Sethoxydim**

Preparation and dilution of formulated sethoxydim were the same as for formulated cycloxydim due to the same content of active ingredient in the formulations of these two compounds. Formulated sethoxydim (36  $\mu$ l) was mixed with water to make 200 ml of a 100  $\mu$ M solution. This solution was diluted successively by 10-fold to 10, 1, and 0.1  $\mu$ M.

### **3.2.2.4 Preparation of Technical Grade Sethoxydim**

Technical sethoxydim lithium salt 11 mg ( $11 \text{ mg} \times 75\% \div 327.5$  (molecular mass) = 25  $\mu$ mole) was dissolved in 25 ml of distilled water to make a 1 mM solution. From this stock solution, dilutions were made to prepare 200 ml of 100, 10, 1, and 0.1  $\mu$ M concentrations of sethoxydim.

## **3.2.3 Herbicide Treatments**

After 3 days germination, seedlings with similar radicle lengths (5 to 7 cm) were selected for treatment. Five seedlings were placed on germination papers saturated with prepared herbicide solutions. The germination papers with seedlings were then rolled up and placed in plastic bags containing various concentrations of herbicide. The end of the plastic bag was loosely folded and secured with a paper clip to reduce evaporation. These plastic bags were placed in trays. Trays were covered with aluminum foil and placed in an incubator at 25°C and at an angle of 60°. Seedlings treated with distilled water served as controls.

Seedling lengths were measured under a green safe light every 12 hours. For corn seedlings, measurements were taken on lengths of radicles, mesocotyls, and coleoptiles. For soybean seedlings, measurements on lengths of radicles and hypocotyls were taken. There were two repli-



cations for each treatment. Five seedlings were used for each replication, and the experiment was repeated.

### **3.2.4 Statistical Analysis**

The growth increments of each seedling part measured (radicles, mesocotyls, coleoptiles, hypocotyls) in every treatment bag were averaged. These averaged values were subjected to analysis of variance at every time point examined. Treatment means were separated and compared using either LSD ( $P = 0.05$ ) or Dunnett's procedure ( $P = 0.05$ ).

### **3.3 RESULTS AND DISCUSSION**

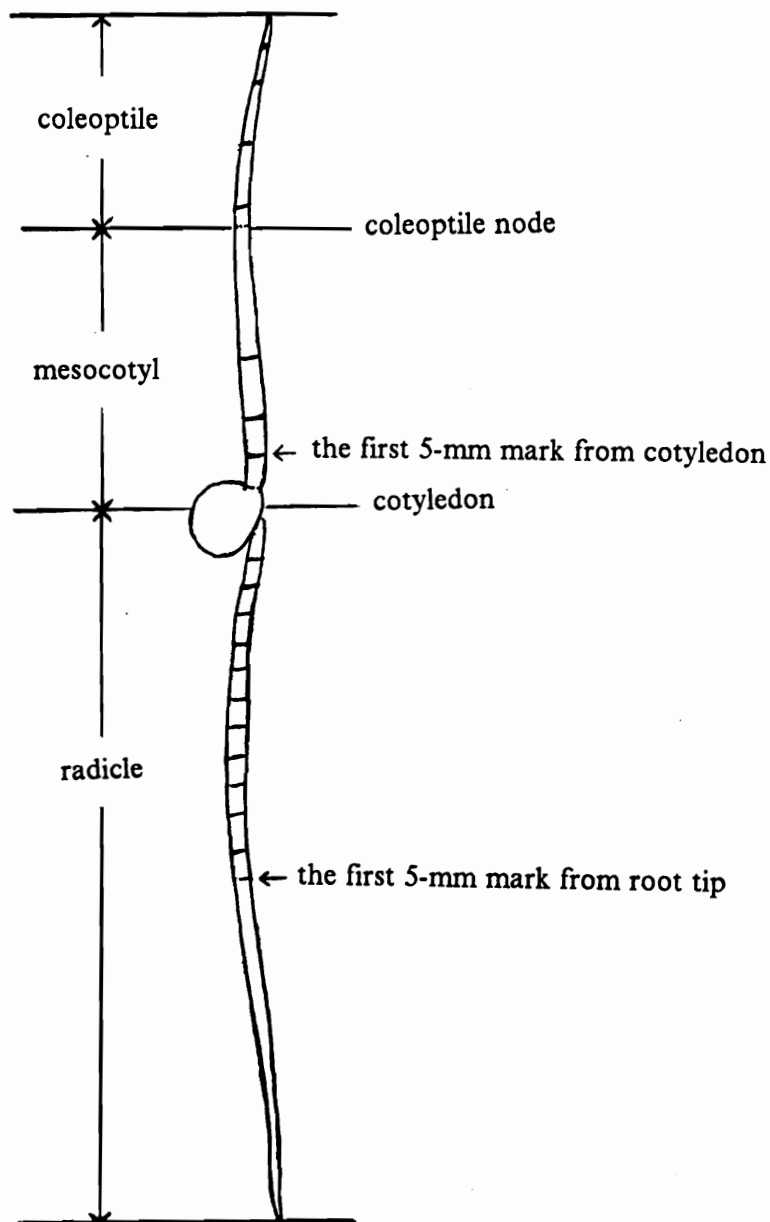
#### **3.3.1 The General Pattern of Seedling Elongation**

The increase in length of a corn seedling during treatments was the result of the elongation of three parts: radicle, mesocotyl, and coleoptile. These three parts increased at different rates during the bioassay period. Lengths of radicle, mesocotyl, and coleoptile in the control group increased approximately 2, 1.2, and 0.8 cm per day, respectively (Table 3-1). Most of the radicle growth was generated from the 2-mm tip of the radicle. The coleoptile node, an area located between the mesocotyl and coleoptile, was responsible for the increase in length of these two seedling parts (Figure 3-1).

#### **3.3.2 Effect of Cycloxydim on Growth of Corn and Soybean Seedlings**

Three patterns of corn radicle growth responding to four levels of cycloxydim concentration were observed. Cycloxydim at 0.1  $\mu\text{M}$ , 1  $\mu\text{M}$ , and 10 or 100  $\mu\text{M}$  concentrations caused various levels of inhibition during the 4-day incubation (Figure 3-2). As shown in Table 3-1, the growth of corn radicles was not affected by cycloxydim at 0.1  $\mu\text{M}$  after the first day of incubation; thereafter radicle growth was inhibited slightly; yet a relatively constant growth rate was maintained throughout the bioassay period. Inhibition of radicle growth of corn treated with 1  $\mu\text{M}$  cycloxydim became visible 2 days after treatment. From then on growth of these radicles was progressively retarded.

Radicles of corn seedlings grew significantly less than controls the first day after treatment with cycloxydim at either 10 or 100  $\mu\text{M}$ . Radicle growth of these seedlings appeared to be inhibited



**Figure 3-1. Diagram of the growth of a corn seedling marked at 5 mm intervals with Indian ink after a 3-day incubation.**

**Table 3-1. Effect of cycloxydim on growth of corn seedlings during 4 days treatment using the rag-doll technique.**

Seedling part	Cycloxydim concentration	Incubation time (days)			
		1	2	3	4
	$\mu\text{M}$	-----length (cm)†-----			
Radicle	0	2.3 a	4.8 a	7.1 a	8.8 a
	0.1	2.2 a	3.7 b	5.3 b	6.5 b
	1	2.0 a	3.0 b	3.5 c	3.8 c
	10	0.7 b	0.8 c	0.8 d	0.8 d
	100	0.7 b	0.7 c	0.7 d	0.7 d
Mesocotyl	0	1.2 a	2.5 a	3.7 a	4.5 a
	0.1	1.1 a	2.2 a	3.5 a	4.2 a
	1	1.1 a	2.3 a	3.5 a	4.0 a
	10	1.1 a	2.4 a	2.8 b	2.9 b
	100	0.9 a	2.1 a	2.5 b	2.6 b
Coleoptile	0	0.6 a	1.5 a	2.3 a	3.1 a
	0.1	0.7 a	1.6 a	2.5 a	3.3 a
	1	0.6 a	1.3 a	2.1 a	3.0 a
	10	0.6 a	1.4 a	1.9 a	2.7 a
	100	0.7 a	1.3 a	1.8 a	2.3 b

† Numbers are means of four replications. Each replication contains five seedlings. Means in the same column and seedling part followed by the same letter are not significantly different (LSD,  $P = 0.05$ ).

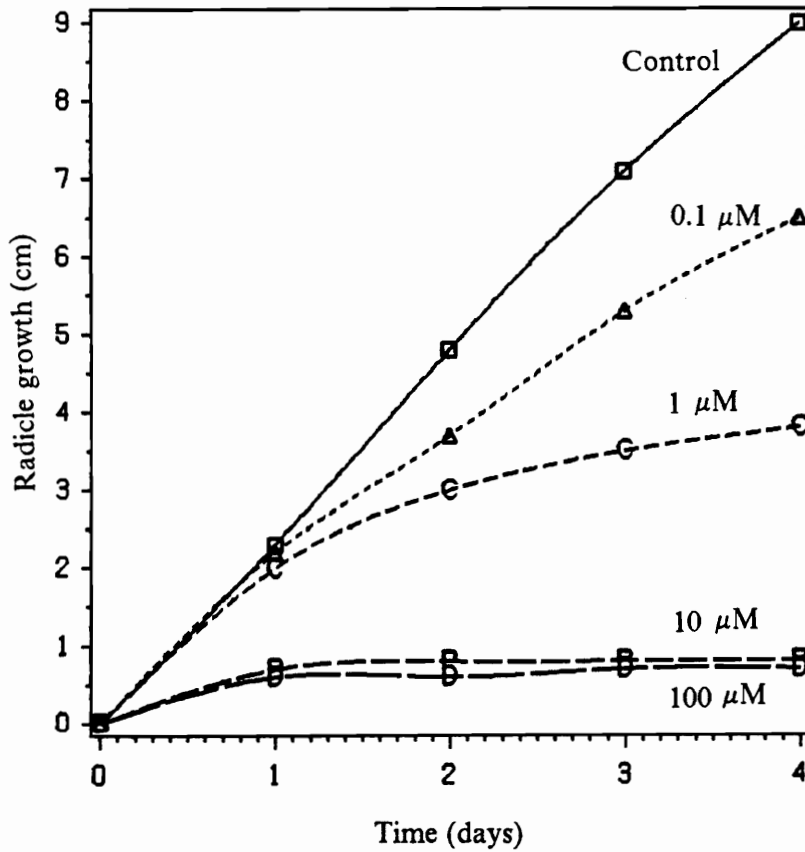


Figure 3-2. Effects of cycloxydim (technical) on radicle growth of corn seedlings examined every 24 hours for 4 days.

completely. Following the cessation of radicle growth, tissue reddening and necrosis developed gradually in the meristematic tips of corn radicles.

Mesocotyl growth was not affected by cycloxydim at 0.1 and 1  $\mu\text{M}$  (Figure 3-3 & Table 3-1). A significant reduction of mesocotyl growth of corn was observed 2 days after treatment with cycloxydim at 10  $\mu\text{M}$  and 100  $\mu\text{M}$ . A significant inhibition of coleoptile growth was observed 3 days after treatment with cycloxydim at 100  $\mu\text{M}$  (Figure 3-4 & Table 3-1). Symptoms similar to those observed in the treated root tips were also observed in the coleoptile nodes of seedlings treated with the highest concentration of cycloxydim after 4 days of incubation. Soybeans were not affected by cycloxydim at 100  $\mu\text{M}$ , the highest concentration used in the bioassays (Table 3-2).

Based on the length of incubation time and the concentration required for the apparent growth inhibition, corn seedling parts affected by cycloxydim in the decreasing order of severity are radicle, mesocotyl, and coleoptile. These different responses of corn seedling parts to cycloxydim may be due to the differences in their morphological structures and nature of growth. The faster growth rate exhibited by roots than shoots may have been due to the higher rate of cell division and elongation in root meristems (Table 3-1). Cells with such high mitotic rates also are high in metabolic activity. Contrasting the growth of shoot and root of corn seedlings, much of the coleoptile length increase during the bioassay was due to cell elongation, which involves a relatively low metabolic activity. In addition, the root system has root hairs which may facilitate the uptake of cycloxydim. Based on these characteristics, roots may therefore accumulate more cycloxydim through uptake, penetration, and translocation within a shorter time than other seedling parts. Root meristematic tissues of etiolated seedlings were the first to show reddening and necrosis. Shoots of corn have slow growth and lack root hairs, which reduces surface area exposed to the chemical; these factors make the coleoptile node a metabolic sink secondary to root meristems. With extended incubation time, coleoptile nodes responded to relatively high concentrations of cycloxydim and developed reddening symptoms as was observed in root meristems.

This study demonstrates clearly that the activity and selectivity of cycloxydim can be expressed in etiolated seedlings, despite the fact that this compound is developed for postemergence use in the field. Some of the postemergence herbicides also exhibit limited preemergence activity (6,10).

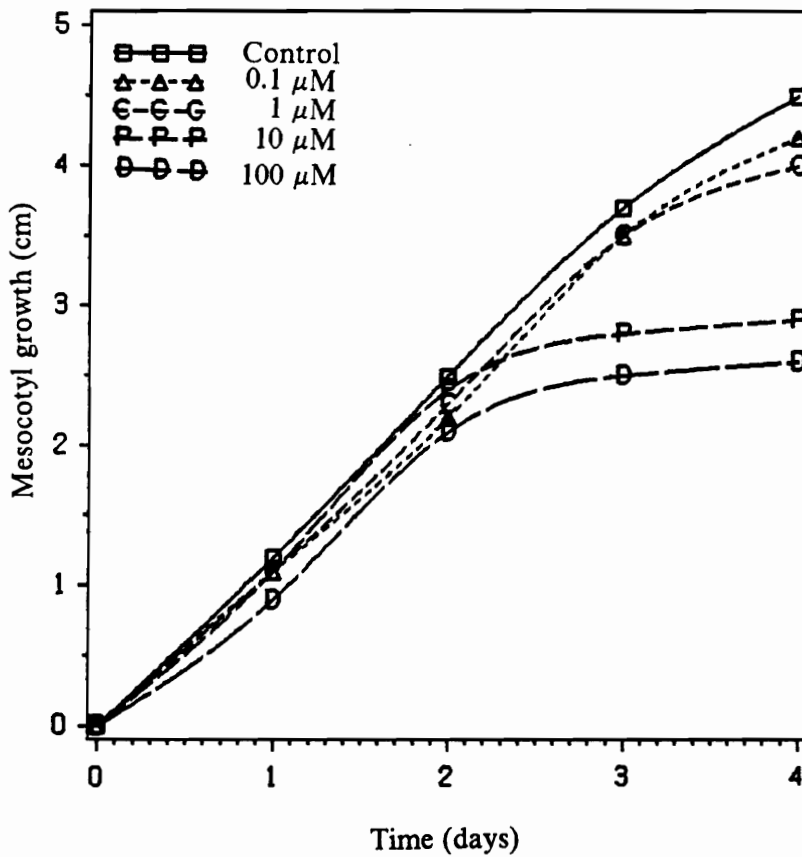


Figure 3-3. Effects of cycloxydim (technical) on mesocotyl growth of corn seedlings examined every 24 hours for 4 days.

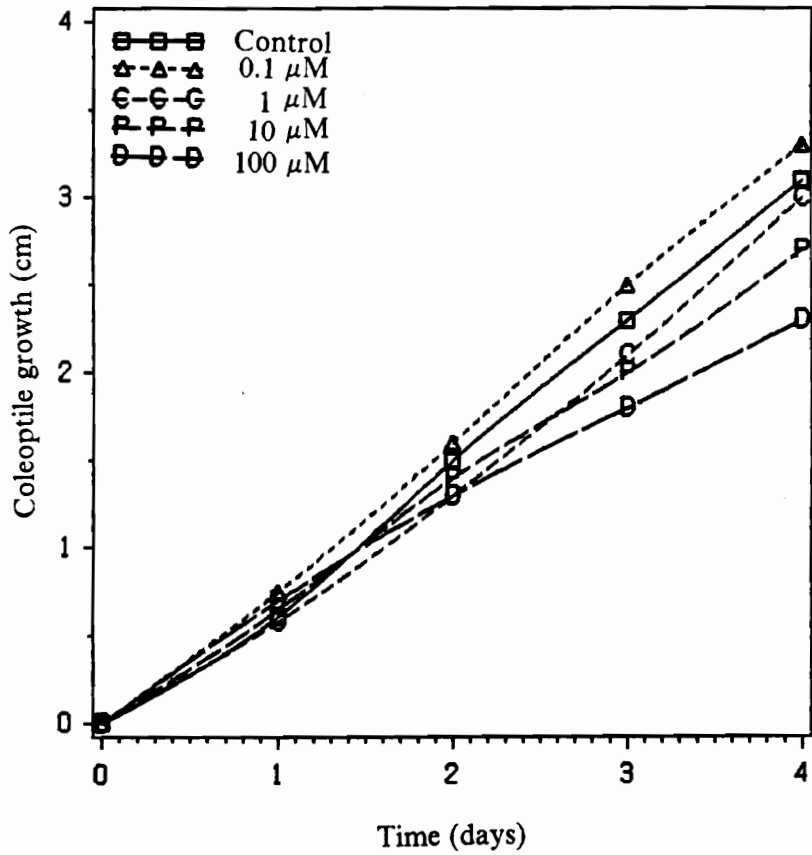


Figure 3-4. Effects of cycloxydim (technical) on coleoptile growth of corn seedlings examined every 24 hours for 4 days.



**Table 3-2. Growth of control and cycloxydim-treated soybean seedlings after 24 and 48 hours incubation.**

Cycloxydim	Seedling part	Hours	
		24	48
$\mu\text{M}$		Growth increment (cm)†	
0	Hypocotyl	2.2	4.8
	Radicle	4.0	8.3
100	Hypocotyl	2.3	4.5
	Radicle	4.2	8.0

† Numbers are means of three replicates. Each replication consisted of at least two seedlings. Analysis of variance (ANOVA) found no significant effect of treatment on the growth of hypocotyls and radicles.

Consistent with results reported by others (1, 2, 3, 8, 10, 11, 13, 17), these data indicate that meristematic tissues are the morphological site of action of cycloxydim.

### 3.3.3 Comparing the Activities of Technical and Formulated Cycloxydim and Sethoxydim

Because of the relatively low sensitivity of shoots and their slowness in response to cycloxydim, parameters such as mesocotyl and coleoptile lengths were considered less suitable than radicle lengths in bioassays. In this comparative study of cycloxydim and sethoxydim, root length was used as a comparative parameter for its rapid and consistent response to these compounds.

Root growth of corn and soybean seedlings treated with four compounds, each at four concentrations, were measured every 12 hours during a 2-day bioassay. Soybean seedlings were not affected by any concentration of these compounds. All compounds at 10  $\mu\text{M}$  inhibited corn root growth 12 hours after treatment (Table 3-3, and Figure 3-5, 3-6, 3-7, 3-8).

Formulated cycloxydim at 1  $\mu\text{M}$  and 10  $\mu\text{M}$  appeared to inhibit radicle growth similarly. Sethoxydim, either formulated or technical, at 1  $\mu\text{M}$  effectively stunted radicle growth 12 hours after treatment, similarly to formulated sethoxydim at 10  $\mu\text{M}$ . However root length still increased at the rate of 0.1 cm per day afterward. Formulated sethoxydim and cycloxydim and technical sethoxydim at 0.1  $\mu\text{M}$  behaved similarly in the inhibition of radicle growth of corn. Radicle lengths of seedlings were significantly reduced 24 hours after treatments and progressively retarded thereafter. Cycloxydim at 0.1  $\mu\text{M}$  caused a significant reduction of radicle length 36 hours after treatment.

Based on the time and herbicide concentrations required for significant reduction of radicle growth, technical sethoxydim appeared to be more potent than technical cycloxydim, and both formulated herbicides were more potent than the technical compounds. Cycloxydim and sethoxydim are known to be degraded by light. The lower activity showed by the technical

**Table 3-3. Effect of formulated and technical grade cycloxydim and sethoxydim on radicle growth of corn seedlings.**

Treatment†	Incubation time (Hours)			
	12	24	36	48
$\mu\text{M}$	-----Growth increment (cm)‡-----			
0	1.1	2.3	3.7	4.8
CT				
0.1	1.1	2.3	3.3 *	3.7 *
1	1.2	2.0	2.8 *	3.0 *
10	0.7 *	0.8 *	0.8 *	0.8 *
ST				
0.1	1.1	1.9 *	2.2 *	2.4 *
1	0.8 *	0.9 *	1.0 *	1.1 *
10	0.6 *	0.6 *	0.6 *	0.6 *
CF				
0.1	1.0	1.5 *	1.9 *	2.2 *
1	0.6 *	0.7 *	0.7 *	0.7 *
10	0.6 *	0.6 *	0.6 *	0.6 *
SF				
0.1	1.0	1.8 *	2.1 *	2.1 *
1	1.0 *	1.2 *	1.4 *	1.4 *
10	0.6 *	0.7 *	0.7 *	0.7 *

† CT: cycloxydim technical. ST: sethoxydim technical.  
CF: cycloxydim formulated. SF: sethoxydim formulated.

‡ Means in the same column followed by an asterisk are significantly different from control according to Dunnett's procedure ( $P = 0.05$ ).

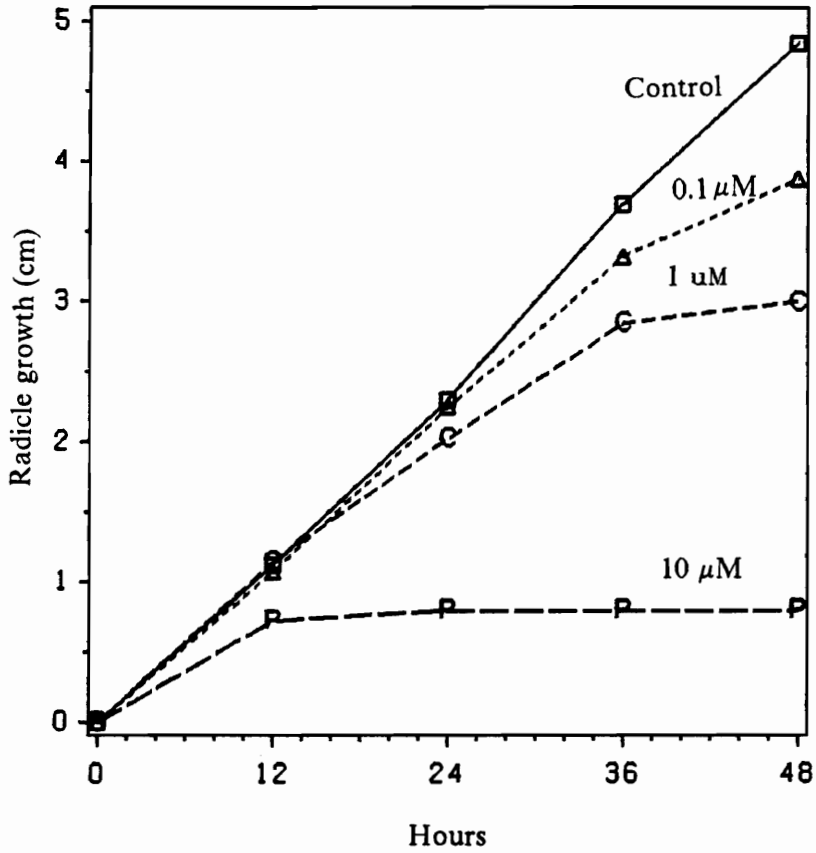


Figure 3-5. Effects of cycloxydim (technical) on root growth of corn seedlings examined every 12 hours for 2 days.

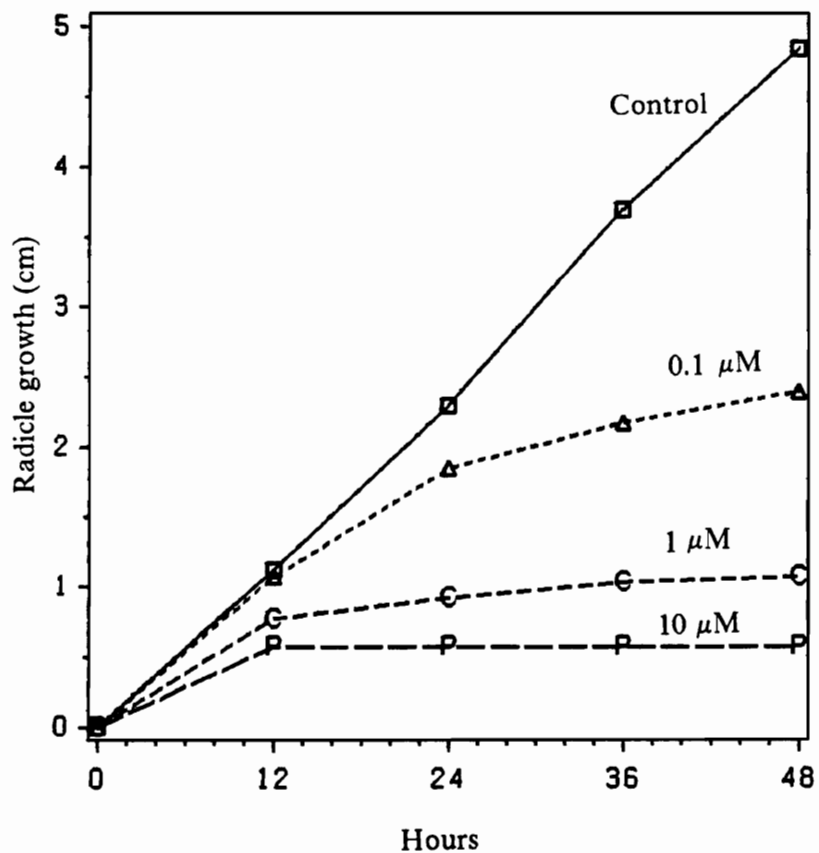


Figure 3-6. Effects of sethoxydim (technical) on root growth of corn seedlings examined every 12 hours for 2 days.

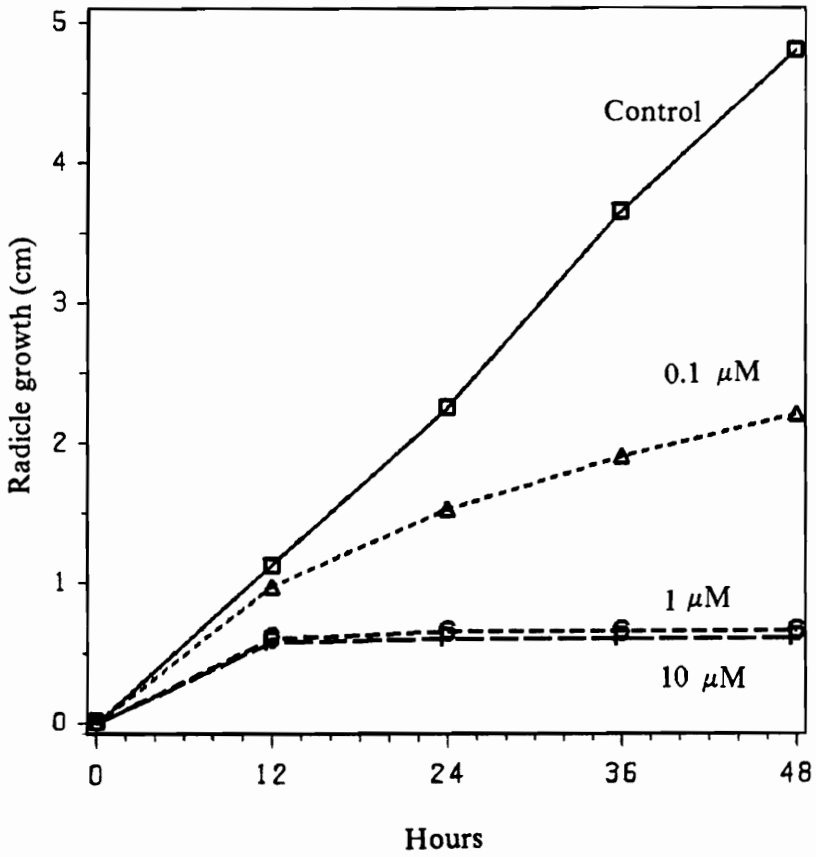


Figure 3-7. Effects of cycloxydim (formulated) on root growth of corn seedlings examined every 12 hours for 2 days.

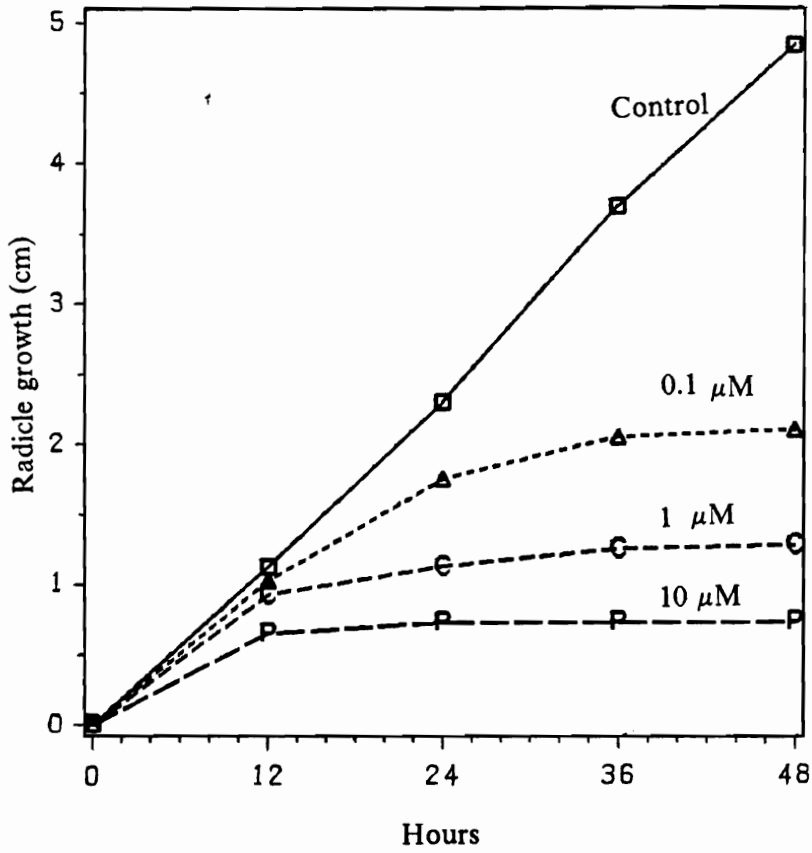


Figure 3-8. Effects of sethoxydim (formulated) on root growth of corn seedlings examined every 12 hours for 2 days.

cycloxydim than sethoxydim technical may be due to some light degradation during storage. The technical grade of cycloxydim used for this bioassay was released from BASF earlier (1985) than was sethoxydim technical (1987).

The relatively higher activity of the formulated compounds may be attributed to the adjuvants present in the formulation. Adjuvants may stabilize the herbicidal compounds to prevent their degradation. Adjuvants may also enhance the uptake of the herbicides into plant tissues. Although the phytotoxicity of the adjuvants cannot be ruled out, the tolerance of soybean seedlings shown at high concentration (100  $\mu$ M) of cycloxydim indicates that the adjuvants are relatively inert.

In conclusion, the seedling bioassay described appears to be very sensitive. The activities of cycloxydim and its related compounds can thus be determined rapidly. In addition, this procedure is simple and economical to set up in a laboratory. Similar to other postemergence compounds reported recently, cycloxydim appears to affect meristematic tissues specifically. Results of this bioassay suggest that cycloxydim is active as a metabolic inhibitor in susceptible plants. Plant materials used in this bioassay are achlorophyllous and thus have no photosynthetic activity. The impaired chlorophyll level and its related photosynthesis reported by other researchers after treatment with compounds having similar herbicidal activity to that of cycloxydim, may result from an indirect effect of these compounds (3, 4, 15). High concentrations of cycloxydim and sethoxydim suppressed radicle growth. An initially slow, then sudden, increase of the inhibitory effects of low concentrations of cycloxydim may indicate the accumulation of this compound in the treated tissue over time. Root elongation was a reliable parameter for detecting the activity of either technical grade or formulated cycloxydim and sethoxydim. This bioassay with root length as an indicator may be extended for use in determining the activities of cycloxydim-related compounds and in determining their selectivities among various plant species.



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# 4.0 EFFECT OF CYCLOXYDIM ON THE GROWTH OF EXCISED CORN AND SOYBEAN ROOT TISSUES

## 4.1 INTRODUCTION

Cycloxydim and other structurally similar cyclohexen-ones and structurally distinct polycyclic alkanolic acids (PCAs) are postemergence herbicides recently developed for selective grass weed control in dicotyledonous crops. In spite of the differences in chemical structures, these compounds have similar herbicidal activity and weed control spectra and cause similar symptoms in susceptible plants. Although the root system is not the application target of these herbicides, the root systems of susceptible plants have been consistently sensitive to them (1, 2, 3, 4, 8, 9, 13, 15).

Hosaka and Takagi (9) first demonstrated the selectivity of sethoxydim and its dose-dependent growth inhibition of susceptible plant species using excised root tips. This tissue-culture system with excised root tips is aseptic, uniform, and sensitive. Since root tips are high in metabolic ac-

tivity, excised root tips have been favored for use when studying the morphological, cytological, physiological, and biochemical effects and mechanism of uptake of sethoxydim (1, 8, 9, 10, 11, 13) and some representative PCAs (5, 6, 7, 12, 15).

The objectives of this paper are:

1. to use excised root tips in tissue culture, a technique reported by Hosaka and Takagi (9), as a bioassay to examine the selectivity of cycloxydim, and
2. to further identify a range of concentrations of cycloxydim for susceptible species for use in later metabolic studies.

## **4.2 MATERIALS AND METHODS**

### **4.2.1 Plant Materials**

Corn kernels and soybean seeds were surface-sterilized with 33% commercial bleach (1.25% sodium hypochlorite) for 15 minutes. Seeds were then rinsed with sterilized distilled water and soaked in distilled water. After the seeds were fully imbibed, they were surface-sterilized again with 33% commercial bleach solution for 15 minutes. Seeds were transferred to a laminar flow hood for rinsing with sterile distilled water. They were then placed on moistened germination papers in pans using aseptic technique. The pans were covered with aluminum foil, and placed in an incubation chamber for germination at 25°C.

### **4.2.2 Root Tip Excision and Tissue Culture**

The procedure for culturing root tips followed Hosaka and Takagi's method (9) with some modifications as explained. After 3 days, 1-cm root tips were excised from seedlings under White's medium (pH 6.0) in a petri dish. Ingredients of the medium are listed in Table 4-1. Root tips were then transferred to a flask and rinsed with fresh medium three times. The flask containing root tips and medium were placed in a shaker with constant shaking (120 cycles/minute) at 26°C to avoid root curvature due to geotropism.

**Table 4-1. White's medium (A basal root culture medium pH 6.0).**

Chemicals	mg/liter
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	200.0
MgSO <sub>4</sub> ·7H <sub>2</sub> O	360.0
KCl	65.0
KNO <sub>3</sub>	80.0
NaH <sub>2</sub> PO <sub>4</sub> ·4H <sub>2</sub> O	16.5
Na <sub>2</sub> SO <sub>4</sub>	200.0
H <sub>3</sub> BO <sub>3</sub>	1.5
MnSO <sub>4</sub>	4.5
KI	0.75
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	1.5
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	2.5
Glycine	3.0
Nicotinic acid	0.5
Pyridoxine hydrochloride	0.1
Thiamine hydrochloride	0.1
Sucrose	20,000.0

### 4.2.3 Preparation of Cycloxydim for Treatments

Stock solutions of technical grade cycloxydim and sethoxydim were prepared. The dilutions were prepared similarly to those described in Chapter 3 except that all solutions were prepared using White's medium rather than distilled water. Volumes of 100 ml of 0.1, 1, and 10  $\mu$ M solutions were prepared for technical grade cycloxydim and sethoxydim lithium salt.

### 4.2.4 Cycloxydim Treatments for Root Tips

For herbicide treatment, excised root tips were transferred to autoclaved KAPAK pouches, which are polyester plastic bags with polyethylene lining (Fisher Scientific Co.), containing 20 ml White's medium with various herbicide concentrations. Two root tips were used in each bag, and each bag was a replicate. The bag was closed by folding the edge of the bag several times and secured with paper clips. For culturing, the folded edges of two bags were attached and the bags were laid flat in a pan so that the folds were pointed up and the bags were laid opposite to each other. This procedure allowed better aeration of the root tips with the air trapped inside the bag and prevented spilling of medium. The pan with bags was placed on a shaker with constant shaking (120 cycles/min) at 26°C. Root tips lengths were measured and recorded at 12, 24, 36, and 48 hours after the commencement of herbicide treatments. Experimental treatments were arranged in a completely randomized design. Every treatment was replicated three times and the experiment was repeated.

## **4.3 RESULTS AND DISCUSSION**

### **4.3.1 Changes in the Morphology of Root Tips in Tissue Culture with Cycloxydim**

Excised root tips of both corn and soybean under the tissue culture conditions grew at approximately 1 cm per day (Tables 4-2 and 4-3). As the length of excised root tip increased, its diameter declined. After 4 days culture, healthy root tips developed numerous lateral roots at and near the cut ends. Soybean root tips were not adversely affected by technical grade cycloxydim and sethoxydim at any concentrations. In contrast, corn root tips treated with cycloxydim showed stunted growth, and the diameters of these root tips remained relatively thick. Many treated corn root tips also showed swollen areas (root primordia) near the cut ends, which were evidence of the suppression of initiation and development of lateral roots in these root tips. Roots exposed to a high herbicide concentration (10  $\mu$ M) developed reddish brown bands near the tips.

### **4.3.2 Effect of Cycloxydim and Sethoxydim on the Growth of Excised Corn and Soybean Root Tips**

Growth of corn root tips treated with cycloxydim at 0.1, 1, and 10  $\mu$ M was progressively retarded during the bioassays (Figure 4-1, Table 4-2). Root tips treated with 1 and 10  $\mu$ M cycloxydim were significantly shorter than the controls 12 hours after treatment. Root tips treated with 1 and 10  $\mu$ M cycloxydim continued to grow approximately 1 mm and 1 mm during the next 12 hours of the first day and the second day, respectively. A significant reduction of root length of root tips treated with 0.1  $\mu$ M cycloxydim was observed 24 hours after the treatment.



**Table 4-2. Effect of technical grade cycloxydim (CT) and sethoxydim (ST) on growth of excised corn root tips using a tissue culture technique.**

Treatment	$\mu\text{M}$	Incubation time (hours)		
		12	24	48
		Growth increment (cm)†		
	0	0.61 a	1.06 a	2.20 a
CT	0.1	0.59 a	0.91 b	1.26 b
	1	0.54 ab	0.63 d	0.76 cd
	10	0.45 c	0.56 d	0.64 de
ST	0.1	0.56 ab	0.76 c	0.89 c
	1	0.47 bc	0.57 d	0.63 de
	10	0.43 c	0.56 d	0.57 e

† Numbers are means of four replicates.  
 Each replication consisted of two excised root tips.  
 Means in the same column followed by the same letter  
 are not significantly different (LSD,  $P = 0.05$ ).

**Table 4-3. Effect of technical grade of cycloxydim (CT) and sethoxydim (ST) on growth of soybean root tips using a tissue culture technique.**

Treatment	$\mu\text{M}$	Incubation time (hours)	
		24	48
		Growth increment (cm)†	
	0	1.0 ab	1.78 b
CT	1	0.95 b	1.82 b
	10	1.15 a	2.11 a
	100	0.93 b	1.68 b
ST	100	0.98 b	1.78 b

† Numbers are means of four replicates.  
 Each replication consisted of two excised root tips.  
 Means in the same column followed by the same letter  
 are not significantly different (LSD,  $P = 0.05$ ).

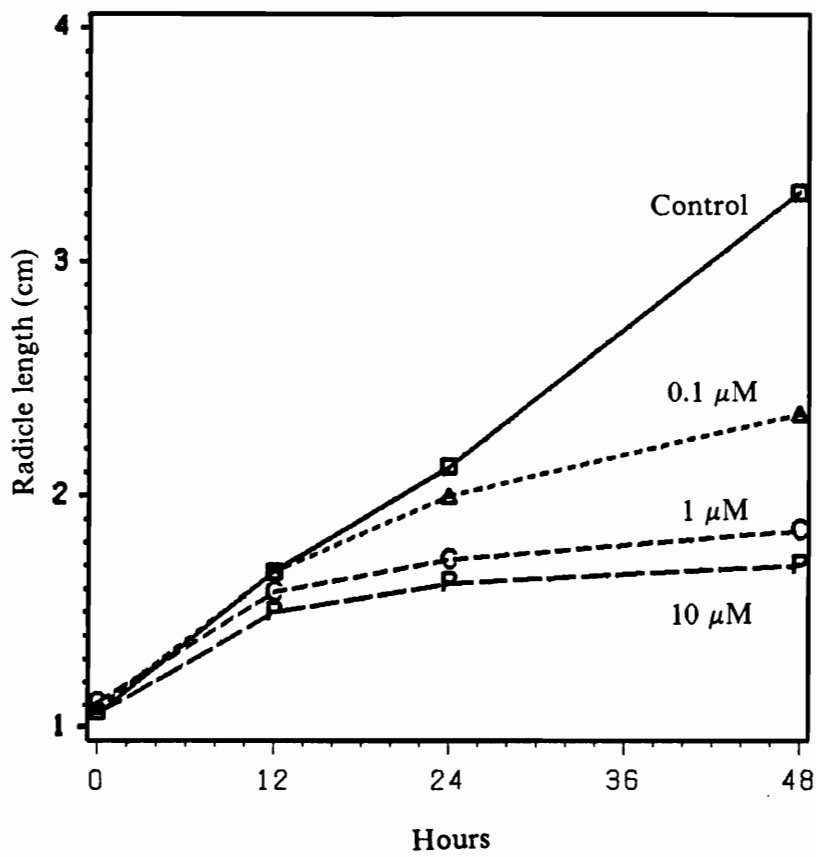


Figure 4-1. Effects of cycloxydim (technical) on growth of corn root tips in tissue culture.

Sethoxydim at 0.1, 1, and 10  $\mu\text{M}$  inhibited corn root growth 12 hours after treatment (Figure 4-2, Table 4-2). Root tips treated with 1 and 10  $\mu\text{M}$  sethoxydim were significantly shorter than the controls. Root tips treated with 0.1, and 1  $\mu\text{M}$  grew approximately 1 mm and 1 mm during the later 12 hours of the first day and the second day, respectively. Root tips treated 10  $\mu\text{M}$  cycloxydim grew approximately 1 mm during the later 12 hours of the first day, and their growth ceased almost completely thereafter. Sethoxydim appeared to be more potent than cycloxydim, because sethoxydim (10  $\mu\text{M}$ ) inhibited root growth completely earlier than cycloxydim at the same concentration.

Soybean root tips were not affected by the technical grade of either sethoxydim or cycloxydim at 100  $\mu\text{M}$ . Growth of soybean root tips appeared to be stimulated with the treatment of technical grade cycloxydim at 10  $\mu\text{M}$  (Table 4-3). This stimulation effect is unexplained. Soybean root tips were not adversely affected by formulated sethoxydim at concentrations as high as 10  $\mu\text{M}$  (Table 4-4). A stimulation of growth was observed with roots treated with that concentration. Growth of soybean root tips was severely inhibited by formulated sethoxydim at 100  $\mu\text{M}$  concentration 12 hours after treatment.

Based on the localization of injury and the inhibition of growth of the root tips, cycloxydim appeared to cause the same adverse effects on excised root tips as it did on whole seedlings. However, the effect of cycloxydim and sethoxydim on excised root tips appeared more rapidly and at lower concentration than with radicles of corn seedlings in the previous bioassays. The lower sensitivity of intact seedlings than that of excised root tips to cycloxydim may be due to the partitioning of the herbicidal compounds among meristematic tissues in intact seedlings. An excised root tip has one meristem, which is the only metabolic sink for the accumulation of cycloxydim. Another possible explanation for the greater sensitivity of excised root tips is that they were bathed in the herbicide solution whereas uptake by the whole seedlings was from the saturated germination paper (a rag-doll technique).

Root tip culture has long been recognized as a technique "ideally suited for studies of the effect of various compounds on root growth" (14). Hosaka and Takagi (9) first reported using this technique for studying the effects of sethoxydim. This technique also was demonstrated here to

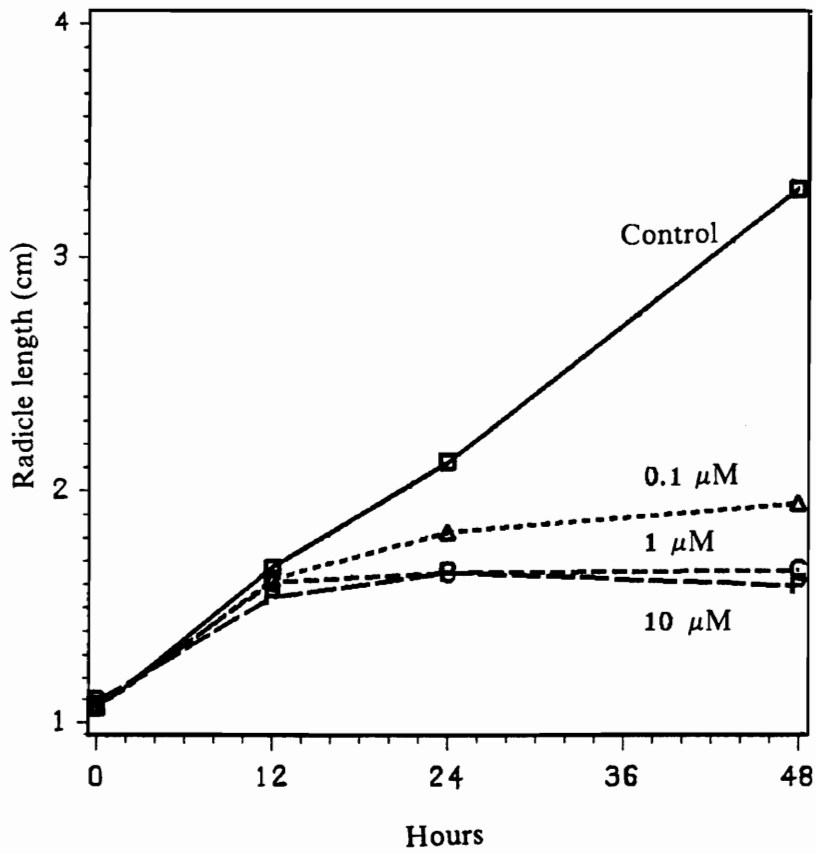


Figure 4-2. Effects of sethoxydim (technical) on growth of corn root tips in tissue culture.

**Table 4-4. Effect of sethoxydim (formulated) on growth of soybean root tips using tissue culture technique.**

Sethoxydim concentration	Incubation time (hours)	
	24	48
$\mu\text{M}$	Growth increment (cm)†	
0	0.78 b	1.66 b
1	0.86 b	1.80 b
10	1.05 a	2.37 a
100	0.15 c	0.15 c

† Numbers are means of four replicates.  
 Each replication consisted of two excised root tips.  
 Means in the same column followed by the same letter are not significantly different (LSD,  $P = 0.05$ ).

be a sensitive one for determining the activity of cycloxydim. Based on these results, cycloxydim and sethoxydim appear to have very similar selectivity and inhibitory effects on root growth of corn. This technique may therefore be extended for bioassays of many postemergence herbicides that have similar herbicidal activities and perhaps may be extended for detecting the susceptibility of other plant species or varieties of interest.

The use of this technique is not recommended for detecting activities of formulated compounds. Soybean (the tolerant species) root tips, which were not affected by technical grade sethoxydim and cycloxydim at 100  $\mu\text{M}$ , showed susceptibility to formulated sethoxydim at the same concentration. This inhibitory effect may be due to phytotoxic additives in the formulation, and/or their enhancement of herbicide uptake. Due to the presence of an open wound in the excised root tip, an excised root tip may lose its selective permeability for compounds in the culture medium. A mass penetration and/or diffusion of additives in the formulation may interfere or mask the selectivity of the herbicides during treatment.

Although the root culture technique appears to be sensitive and yields consistent and uniform results, its success requires stringent aseptic technique and work environment. Results from the use of this technique were similar to those obtained in the intact seedling bioassays. If an individual is only interested in examining a general trend of activity of a compound or the susceptibilities of plant species, the bioassay using seedlings may be less time consuming and less tedious to perform and yet provide acceptable results.

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## 5.0 HISTOLOGICAL EFFECTS OF CYCLOXYDIM ON CORN RADICLES

### 5.1 INTRODUCTION

Cycloxydim is a potent postemergence herbicide recently developed for grass weed control in dicotyledonous crops. The high sensitivity of meristematic tissues of susceptible plants to cycloxydim has been demonstrated in field application (1) and laboratory bioassays (data in chapter 3). The spectrum of weed control and symptom development in susceptible plants caused by cycloxydim are similar to those for other cyclohexen-ones and polycyclic alkanolic acids (PCAs) (1, 2, 5, 6, 7, 10). The strong inhibitory effect of these herbicides on growth of susceptible plants and the high sensitivity of meristematic tissues have led researchers to examine their effects as possible mitotic inhibitors on morphology, histology, and cytology of meristem tissues (3, 4, 5, 6, 7, 8, 9, 10).

Jain and Vanden Born (6) reported that two PCAs, haloxyfop and fluazifop, and a cyclohexen-one, sethoxydim, caused similar morphological injuries in developing wild oat (*Avena fatua* L.) stems after foliar application. They related the inhibition of cell division and elongation

caused by these herbicides to the cessation of growth and the subsequent development of necrosis in the nodal areas of stems. Swisher and Corbin (9) reported the appearance of disorganized apical regions and necrotic cells in leaf primordia and apices of shoots and roots of johnsongrass [*Sorghum halepense* (L.) Pers.] after foliar application of sethoxydim. Hosaka and his colleagues studied the effects of sethoxydim on corn seedlings (4, 5). They reported that "the histological damage to corn root tips treated with sethoxydim was observed in the cytoplasm first". They observed vacuolated cells and aggregation of the cytoplasm as well as cells with apparent mitosis in corn root tips treated with 0.1  $\mu$ M sethoxydim 48 hours after treatment. Similar vacuolization of meristematic cells was observed by Vaughn and Merkle (10) in sorghum [*Sorghum bicolor* (L.) Moench.] treated with haloxyfop.

Consistent with the finding reported by Asare-Boamah and Fletcher (3), Hosaka et al. (4) observed binucleate cells in sethoxydim-treated root tips. As suggested by Asare-Boamah and Fletcher, sethoxydim might induce "the formation of binucleate cells by interfering with the normal function of microtubules and subsequently prevent the formation of cell-plate and -wall formation between divided nuclei". Based on the cytological examination of mitotic cells in control and treated root tips, Asare-Boamah and Fletcher reported that sethoxydim appeared to "interfere with cytokinesis and not with karyokinesis".

Hosaka and Takagi (5) also examined the effects of sethoxydim on mitotic activity of meristematic cells of corn root tips with the use of  $^{14}\text{C}$ -thymidine, a precursor for the synthesis of nucleic acids. They reported that the inhibition of DNA synthesis did not directly contribute to the inhibition of cell division, because DNA synthesis was inhibited after growth inhibition was observed. Kim and Bendixen (7) used  $^3\text{H}$ -thymidine and -uridine and  $^{14}\text{C}$ -leucine to examine the effects of two PCAs, haloxyfop and CGA-82725 {2-propynyl-2-[4-(3,5-dichloro-2-pyridinyl)oxy]phenoxy]propanoic acid}, on cell division, cell cycle dynamics, and nucleic acid and protein synthesis in oat root tips in order to characterize growth inhibition caused by them. The  $^{14}\text{C}$ -leucine incorporation was inhibited at the earliest and at the most severe level among labeled compounds used in the study. They concluded that both herbicides inhibited cell division by inhibiting protein synthesis in the  $G_2$  stage of interphase.

Cycloxydim is structurally different from PCAs, but is structurally similar to sethoxydim, with a cyclohexanedione ring common to both compounds (Figures 2-1 and 2-2). Only one of the several side groups attached to the common ring is different between the two compounds. In sethoxydim, this side group is an open chain containing five carbons and sulfur, whereas in cycloxydim this side group is a thio-pyran ring. It is not known whether this difference in structure of cycloxydim contributes any unique herbicidal activity at the cell and tissue levels. No morphological and histological studies of cycloxydim on sensitive plant tissues have been reported. Histological studies of cycloxydim may be used as a probe for investigating its mode of action and/or any unique herbicidal effects. Thus the objective of this paper is to examine the morphological effect of cycloxydim on corn root tips at the tissue and cell levels.

## **5.2 MATERIALS AND METHODS**

### **5.2.1 Preparation of plant materials**

Corn plant preparation, which included seed imbibition, surface sterilization, and germination was the same as that described previously in Chapters 3 and 4, as were the preparation of cycloxydim (formulated) concentrations of 0.1, 1, and 10  $\mu\text{M}$  and the subsequent cycloxydim treatment to the seedlings. After treatment with various concentrations of cycloxydim for 24 hours, corn seedlings were rinsed with distilled water and blotted dry on paper towels. Root tips 0.2 cm long were excised from the seedlings and were subjected to microtechnique preparation reported by Swisher and Corbin (9) with modification. The preparation included prefixation, postfixation, dehydration, sectioning and staining.

### **5.2.2 Prefixation and postfixation**

Root tips were prefixed in 3% glutaraldehyde buffered to 0.1 M at pH 6.8 with Sorensen's phosphate buffer for 24 hours at 4°C (Dr. Randolph L. Grayson, personal communication). After prefixation, root tips were rinsed with Sorensen's buffer three times. Tips were swirled around in the container for 15 minutes during each rinsing. Root tips were then postfixated in 1% osmium tetroxide for 3 hours. Root tips were then rinsed with buffer for three times (15 minutes for each rinsing).

### **5.2.3 Dehydration, infiltration, and embedding**

Root tips were first rinsed three times in distilled water for 5 minutes each. Root tips were then dehydrated in a graded ethanol series involving treatment for 5 minutes each in 15%, 25%, 40%, 50%, and 60% ethanol, consecutively, and 10 minutes in 70%, 80%, and 95% consecutively. Lastly the tips were dehydrated in a series of three 100% ethanol solutions for 5 minutes each.

Root tips were then infiltrated with Spurr's epoxy resin (Polysciences, Inc., Warrington, PA 18976) through a graded series in acetone. The solutions for the first 2 hours and next two 4-hour periods were 3:1, 1:1, and 1:3 acetone and Spurr's medium, respectively. Root tips were finally embedded in 100% Spurr's medium for 12 hours and then dried in an oven at 60°C overnight.

### **5.2.4 Sectioning**

Longitudinal sections of root tips were sectioned at 3 to 5  $\mu\text{m}$  thickness on an ultramicrotome (Porter-Blume MT2B) using a glass knife. Sections were laid on glass slides.

### **5.2.5 Staining**

Toluidine blue (1%) in 0.5 % sodium borate was used to stain the sections.

### **5.2.6 Viewing**

Slides were mounted on a Leitz microscope equipped with a camera. Photographs of representative sections were taken.

## **5.3 RESULTS**

### **5.3.1 The Anatomy of A Normal Root Tip**

A longitudinal section of a corn root tip is shown in Figure 5-1. The tip is covered by a root cap; and inside the cap, tissues can be further divided into epidermis, cortex, and stele. The stele is composed of cells with high mitotic activity and cells undergoing differentiation. Metaxylem development is evident as cells with large diameter and high levels of vacuolization. Cells in various stages of mitosis are observed in the stelar region. These cells are characterized by dense cytoplasm and dark-stained nuclei. Some of the cells appeared to be daughter cells just produced from either longitudinal or horizontal cell division.

### **5.3.2 Cells in Root Apices 24 Hours after Cycloxydim Treatments**

Cells in the apex of a root treated with 1  $\mu\text{M}$  cycloxydim (Figure 5-2) appeared to possess large vacuoles, which were not commonly seen in cells in this region of roots. Nuclei with dark stained nucleoli or chromatin were present in many of these cells and appeared to be intact. In the root cap, most cells except the ones located at the center lost most if not all of their cytoplasm. In some cells, only nuclei and cell walls remained. Injury to cells at the center appeared to be less severe than to cells located at and near the epidermis.

Anatomical injury in cells of roots caused by 10  $\mu\text{M}$  cycloxydim was more prominent than that in roots treated with 1  $\mu\text{M}$  herbicide (Figure 5-3). All cells in the cortex had little or no cytoplasm. The remaining cell walls and a few nuclei gave a cork-like appearance. Nuclei, when they existed in the cells, appeared to be pressed against the cell walls. Some nuclei still contained

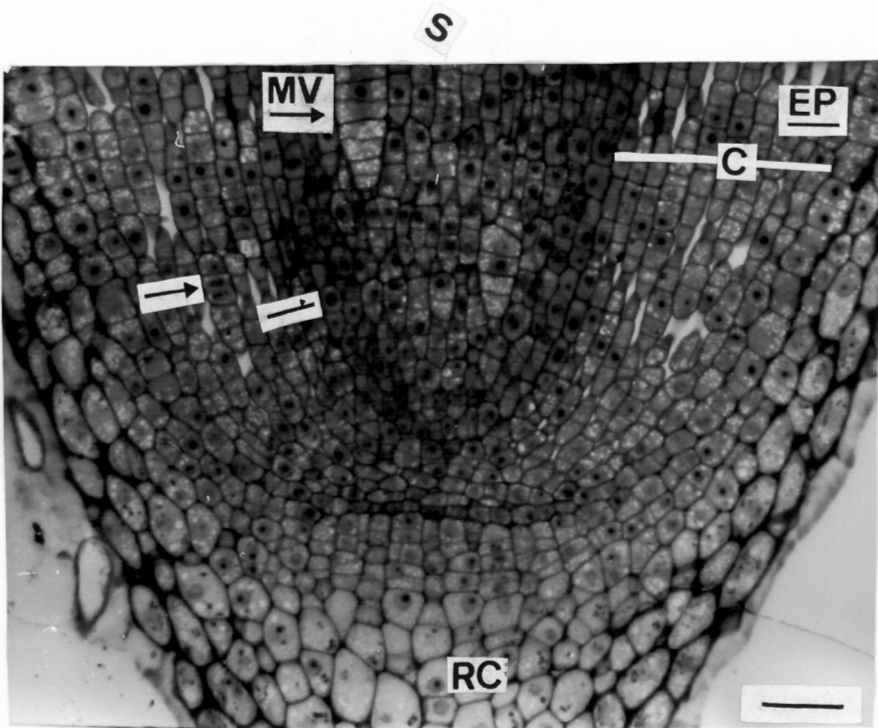


Figure 5-1. The primary root tip of an untreated corn seedling: Root tips showing root cap (RC), epidermis (EP), cortex (C), a stele (S) with immature metaxylem vessels (MV) and cells in mitosis (arrows). Bar = 50  $\mu$ m.

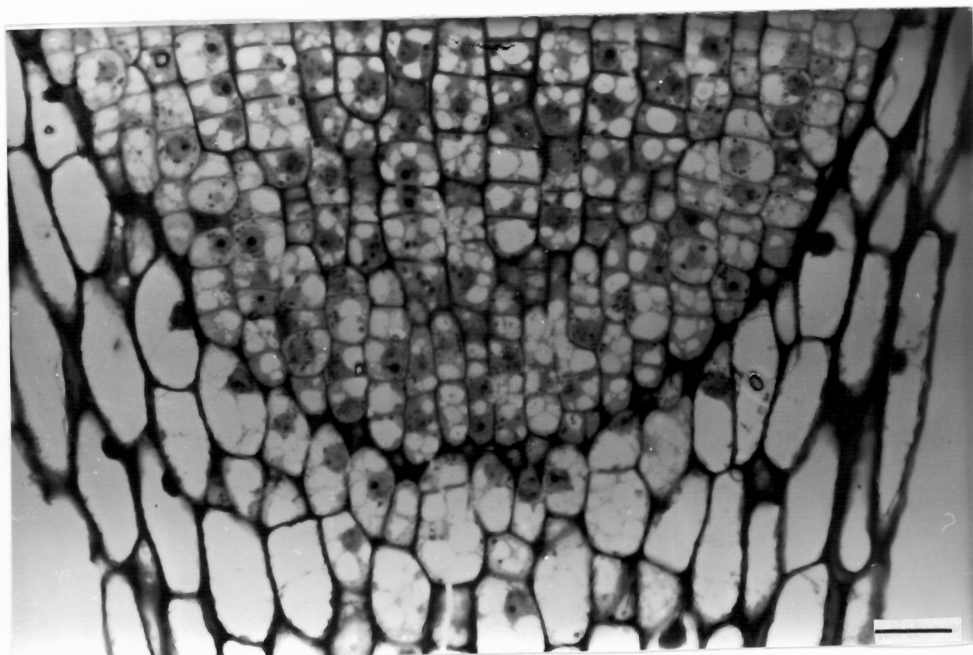


Figure 5-2. Corn root tip 24 hours after treatment with 1  $\mu$ M cycloxydim: Note cells with large vacuoles and cells lacking cytoplasm in root cap. Bar = 25  $\mu$ m.



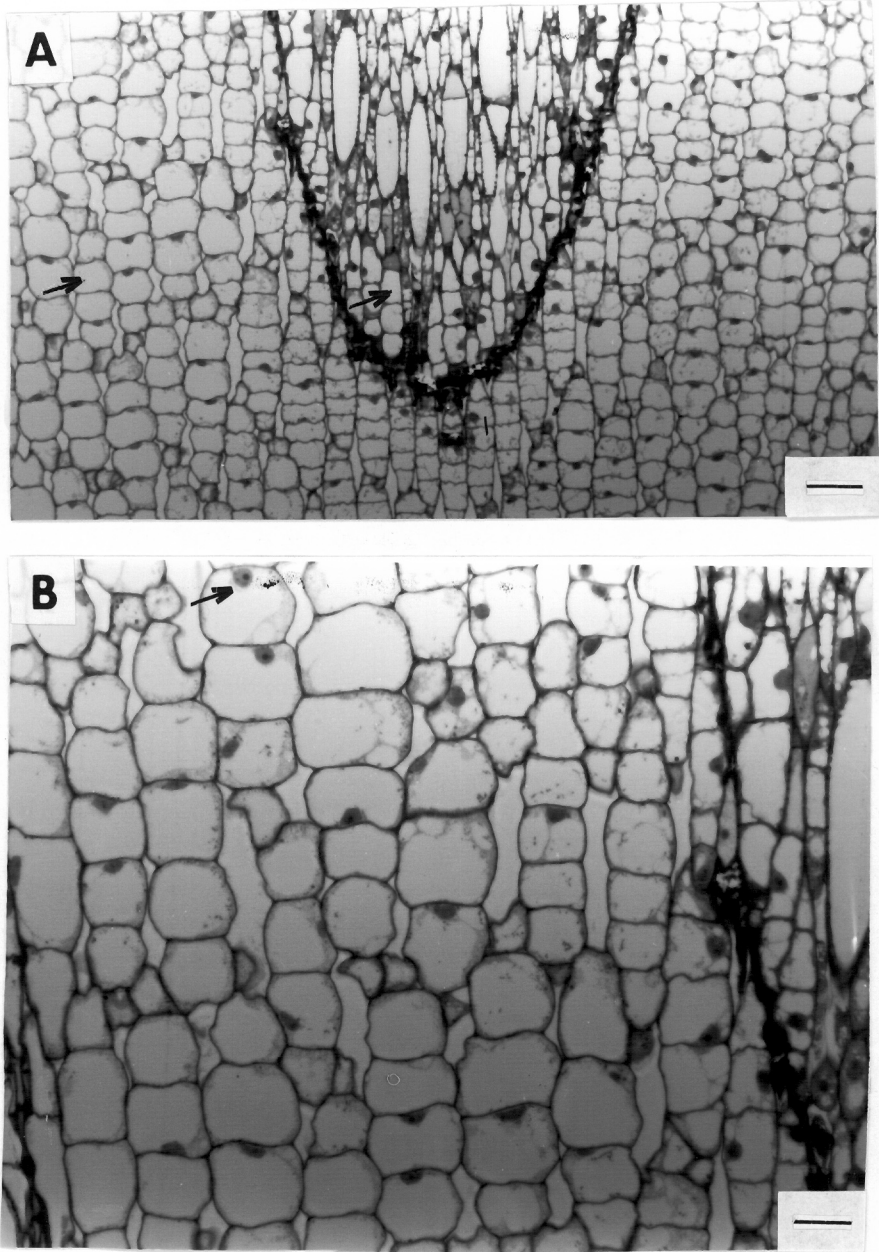


Figure 5-3. Root tip 24 hours after treatment with 10  $\mu$ M cycloxydim: A: Root tip showing cells lacking cytoplasm (arrows) in cortex and stele. Bar = 50  $\mu$ m. B: Close-up of this section showing cells lacking cytoplasm while nuclei appeared to be intact. Bar = 25  $\mu$ m.

nucleoli and appeared to be undisturbed by cycloxydim. Most cells in the stelar region appeared to have no cell contents, except some cells still had nuclei and vacuolated cytoplasm.

### 5.3.3 Cells Located at 1 mm Above the Apices of Corn Roots

Figure 5-4A shows cells located at 1 mm above the apex of an untreated corn root. In roots treated with 0.1  $\mu\text{M}$  cycloxydim, cells 1 mm behind the root tips showed vacuolization similar to the cells at the apices. Cells close to the epidermis possessed larger vacuoles and less cytoplasm than the ones close to and in the stelar region (Figure 5-4B). Pronounced vacuolization was also observed in xylem (metaxylem) cells. Slight vacuolization was observed in cells adjacent to the xylem cells. A number of cell layers located between the metaxylem and cortex appeared to have normal cytoplasm and intact nuclei.

Compared to the cells in roots treated with 0.1  $\mu\text{M}$  cycloxydim, cells in a root treated with 1  $\mu\text{M}$  cycloxydim showed more severe injury (Figure 5-4C). All cells in this region appeared to be affected by the cycloxydim treatment. Cells at or near the epidermis had little or no cytoplasm. Cells located near the center of the root possessed few large vacuoles. A decrease in severity of cellular injury toward the center of the root was evident similarly to that observed in roots treated with 0.1  $\mu\text{M}$  cycloxydim; however, the injury was of greater magnitude. The vacuolization in the metaxylem appeared more severe than that in roots treated with 0.1  $\mu\text{M}$  cycloxydim.

## 5.4 DISCUSSION

After 24 hours exposure to 0.1  $\mu\text{M}$  and 1  $\mu\text{M}$  cycloxydim, vacuolization was evident in epidermal, cortex, and stelar cells in roots. Nuclei in these cells appeared to be unaffected or affected much less than the cytoplasm. The level of vacuolization in root tips increased with in-

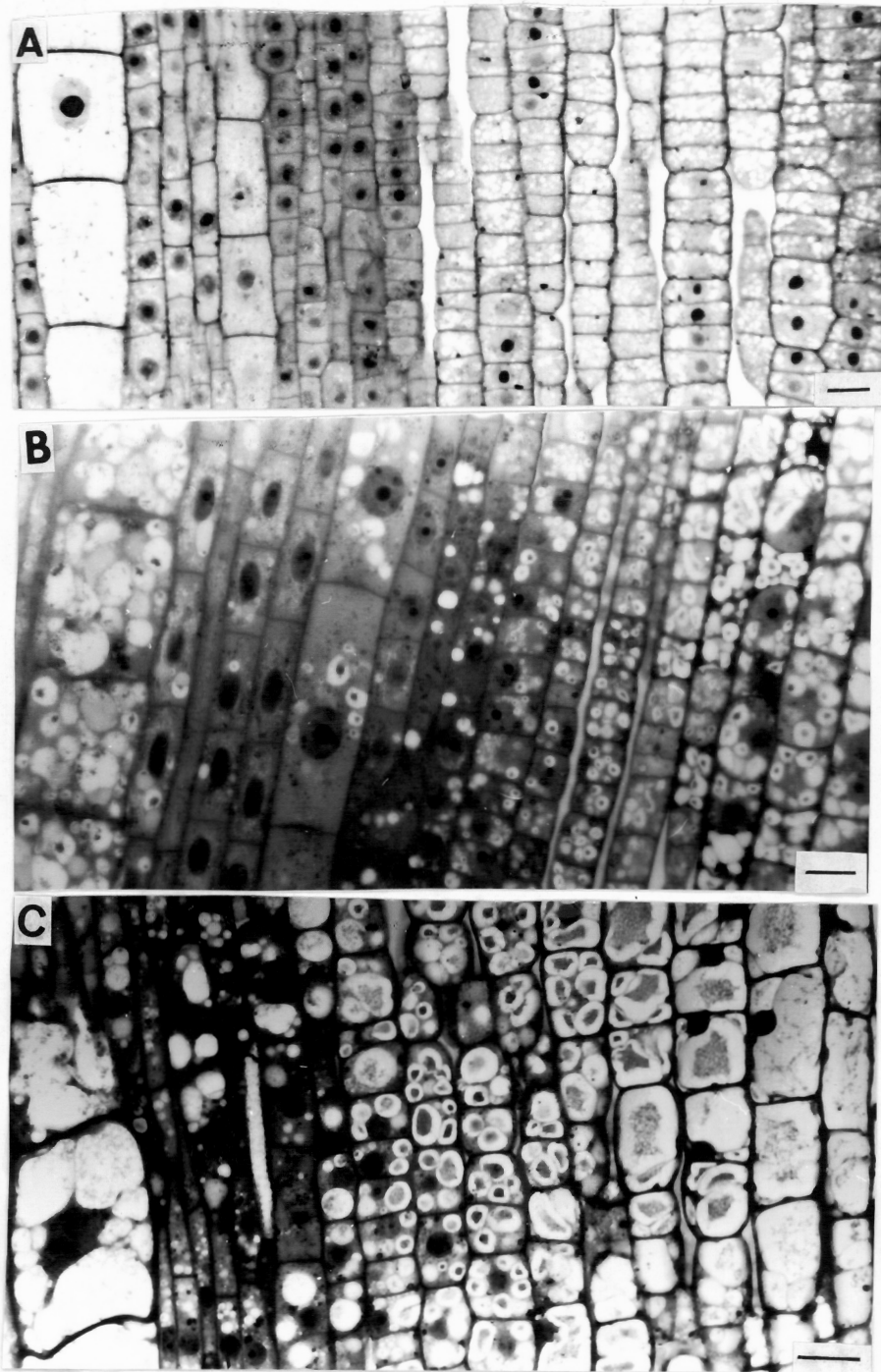


Figure 5-4. Cells at 1 mm above the root apices of untreated and cycloxydim-treated corn roots: A: an untreated root. B: Corn root tip 24 hours after treatment with 0.1  $\mu\text{M}$  cycloxydim showing unusually large vacuoles at (or near) epidermal side and small and numerous vacuoles in cells toward the center of the root. C: Root tip 24 hours after treatment with 1  $\mu\text{M}$  cycloxydim. Bar = 25  $\mu\text{m}$ .

creased rate of cycloxydim. Similar vacuolization has been reported previously for other herbicides with similar herbicidal activity and selectivity, including sethoxydim (4, 5), diclofop (8), and haloxyfop (10). These herbicides, though different in chemical structures, caused similar morphological damage in meristems.

According to Vaughn (10), similar precocious vacuolization of meristem cells has also been noted for compounds different from cyclohexen-ones and PCAs, such as bensulide (*O,O*-bis(1-methylethyl) *S*-[2-[(phenylsulfonyl)amino]ethyl]phosphoro-dithioate), bromacil [5-bromo-6-methyl-3-(1-methylpropyl)-2,4(1*H*,3*H*)pyrimidinedione], and propham (1-methylethyl phenylcarbamate). These compounds differ in chemical structures as well as selectivity. Bromacil, known as an inhibitor of the Hill reaction, is the only one of these three compounds whose primary mode of action has been elucidated. Hosaka et al. (4) reported that trifluralin [2,6-dinitro-*N,N*-dipropyl-4-(trifluoromethyl)benzeneamine], benthioncarb {*S*[(4-chlorophenyl)-methyl]-diethylcarbamothioate}, and propham caused morphological and cytological injuries different from those caused by sethoxydim.

Cells in roots treated with cycloxydim at 0.1 and 1  $\mu$ M showed decreasing levels of injury from the epidermal layer toward the center of the roots. This injury gradient probably reflects the gradient of penetration of cycloxydim into roots. The vacuolization observed in xylem cells and adjacent cells also indicated the possibility of the transportation of cycloxydim through the vascular tissues and subsequent diffusion. However, judging from the number of cell layers affected by the two processes, diffusion from the outside medium into roots appeared to be more prominent of the two.

Cells showed progressive changes responding to the cycloxydim treatments. As the injury level increased, cells changed from having small but numerous vacuoles to cells with a few but large vacuoles, and then to cells appearing empty due to the loss of most cell content. This change in cell anatomy caused by cycloxydim is similar to the sequence of changes in cells undergoing senescence. As stated by Vaughn and Merkle (10), "this sequence of changes in cells involves fusion of small vacuoles into large vacuoles; eventually the tonoplast membrane of the vacuole breaks

down, followed by a complete loss of compartmentation of the cell". Corn roots 24 hours after exposure to cycloxydim apparently changed from their active role in metabolism to senescence.

After 24 hours treatment with 0.1  $\mu\text{M}$  cycloxydim, roots of corn seedlings still increased root length but at a very slow rate. In Figure 5-4B, a few layers of cells located near the center of the root appeared not to be affected by 0.1  $\mu\text{M}$  cycloxydim; maybe these cells still contributed to root elongation. Roots treated with 1  $\mu\text{M}$  cycloxydim and higher had completely ceased growth 12 hours after treatment. As seen in Figure 5-2 and Figure 5-4C, all cells across the section showed damage at severe levels, and were thus unable to contribute to growth.

This study examined the histological effects of cycloxydim at several concentrations on root meristems. The findings of this work resemble those reported by Vaughn and Merkle (10), who examined the effect of haloxyfop at one concentration (1  $\mu\text{M}$ ) over time. This suggested that haloxyfop and cycloxydim at comparable rates caused similar phytotoxic effects on root tips. The resemblance of the injury caused by a high concentration at a shorter time to that caused by low concentration for a longer time indicated strongly that these herbicides accumulated in root meristematic tissues over the incubation period.

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## **6.0 EFFECTS OF CYCLOXYDIM ON <sup>14</sup>C-UL-GLUCOSE AND -ACETATE METABOLISM IN EXCISED CORN AND SOYBEAN ROOT TIPS**

### **6.1 INTRODUCTION**

Cycloxydim is a postemergence herbicide developed recently for its high activity against grass species in dicotyledonous crops. Although structurally different, it produces similar injury symptoms on susceptible plants and has a similar spectrum of selectivity to those of other postemergence cyclohexen-ones and polycyclic alkanolic acids (PCAs) (1, 2, 3, 8, 15, 17, 24). A few days after application of any of these herbicides, susceptible plants exhibit reddening. Meristematic tissues appear to be sensitive to these herbicides and are the first to develop injury symptoms.

Asare-Boamah and Fletcher (3) and Chandrasena and Sagar (7) document various responses exhibited by susceptible plants a few days after application, to sethoxydim and fluazifop (a PCA). These responses include cessation of growth, reduction in length and fresh and dry weights, reduction in respiration and photosynthesis, and increase in levels of sugar and anthocyanin content. The color change of treated susceptible plants apparently signals a shift in carbohydrate metabolism and is related to the increase of sugar and anthocyanin.

Short term (24 to 48 hours) effects of diclofop-methyl on corn root tips were examined by Hoppe (16). He reported that respiration, protein, and nucleic acid content in root tips were not affected by the PCA. In addition, he found an accumulation of alcohol-soluble compounds, reduced phospholipids and triglycerides, and an increase in glycolipids. Gealy and Slife (12) examined the growth and apparent photosynthesis in leaves of corn treated with sethoxydim for 24 to 48 hours. They reported growth inhibition of corn leaves within 24 hours, whereas apparent photosynthesis inhibition in corn leaves did not occur until 24 hours after treatment. They suggested that, due to the late timing of photosynthesis inhibition, photosynthesis could not be the primary site of action of sethoxydim. Gronwald (13) also found that growth inhibition preceded the impairment of respiration and suggested that respiration inhibition was a secondary effect of haloxyfop and haloxyfop-methyl.

Radioisotope techniques have been favored by researchers for use in investigating the modes of action of these postemergence grass-selective compounds. Many researchers have assayed the incorporation of several  $^{14}\text{C}$  precursors into various metabolites in plant materials such as leaves and leaf discs (20), shoots (4), isolated cells (8, 15), chloroplasts (5, 6, 11, 26, 27), coleoptiles and hypocotyls (30), and excised root tips (16, 18, 19, 20, 21, 22, 23, 25) of susceptible and tolerant plants after short-term exposures to these postemergence compounds. Interestingly, sethoxydim and some PCAs, though with distinct chemical structures, showed a similar inhibitory effect on lipid synthesis.

Lipid synthesis is a pathway more sensitive than the synthesis of protein, RNA, and DNA and photosynthesis in plant cells and tissues based on the concentrations and incubation times required for inhibition (15, 17, 21, 22). Among the lipid classes examined in corn root tips treated with



sethoxydim or diclofop-methyl, lipids containing a fatty acid moiety were much reduced but sterol lipids containing no fatty acid were not adversely affected. The latter, in fact, were increased when incubated with either  $^{14}\text{C}$ -UL-glucose or -acetate (4, 18, 19, 20, 23).

Very recently, acetyl CoA carboxylases in leaves and shoots of tolerant and susceptible plants have been reported to have differential sensitivity to cyclohexanediones and PCA compounds (5, 6, 11, 28, 31, 32, 34, 35, 36). This enzyme is the first committed enzyme involved in fatty acid synthesis, and its sensitivity to these compounds correlates well with the inhibition of lipid synthesis reported in the literature. In addition, the PCAs, quizalofop and haloxyfop, were reported to inhibit photosynthetic electron transport in isolated corn chloroplasts and to inhibit two dehydrogenases (DH) involved in glycolysis and the TCA cycle of corn cells (9, 33). However, these inhibitions were caused by these compounds at relatively high concentrations in comparison with the concentrations used to inhibit lipid synthesis.

As an approach for examining mode of action, a few researchers reported the effect of some PCA compounds on the synthesis of compounds involved in different but closely related pathways with the use of one labeled precursor. Cho et al. (8) used corn cells incubated with  $^{14}\text{C}$ -UL-sucrose and haloxyfop in cell-suspension culture. They reported that the incorporation of  $^{14}\text{C}$  into lipids, amino acids, and free sugar was inhibited, enhanced, and not affected by haloxyfop, respectively. Hoppe (20) used leaves removed from diclofop-methyl-treated plants and then incubated these leaves with  $^{14}\text{C}$ -acetate. He reported that inhibition of  $^{14}\text{C}$  incorporation into lipids by diclofop-methyl coincided with increasing  $^{14}\text{C}$  distribution into carboxylic acids and amino acids. In similar experiments with the use of leaf pieces and excised 1-cm root tips, Hoppe reported a similar inhibitory effect of diclofop-methyl on  $^{14}\text{C}$  incorporation into lipids in these tissues. However, the enhancement of  $^{14}\text{C}$  incorporation into water-soluble compounds could not be clearly demonstrated. Radioactivity of the water-soluble fraction of the extracts was variable.

The meristematic tissue responsible for the growth of roots is located within the first 0.2 cm of the root tips, any greater length would include tissues with relatively lower metabolic activity. When using root tips longer than 0.2 cm in radioactive tracer experiments, a dilution effect of a herbicidal compound on metabolic activity is anticipated and may result in variation.

Organisms maintain a well-regulated and balanced metabolism. Many biochemical pathways are interrelated. The inhibition of one pathway may have a direct impact on the functions of the others, resulting in inhibition of some pathways and stimulation in others. The changes occurring in the distribution of  $^{14}\text{C}$  from one labeled precursor into compounds involved in several closely related pathways under the influence of a herbicide may provide a researcher with information on the physiological effect of this herbicide in an organism and may help to pin-point further the site of action. No report on cyclohexen-ones with such scope has been published.

Glucose is the primary carbon source of lipid synthesis and other closely related pathways, such as glycolysis and the TCA cycle. Danks et al. (10) reported using  $^{14}\text{C}$ -UL-glucose to assess the effects of allelopathic phenolic compounds on the synthesis of major glucose metabolites, such as lipids, amino acids, and carboxylic acids in rose (*Rosa* sp.) cells in suspension cultures.  $^{14}\text{C}$ -UL-glucose is incorporated readily into lipids synthesized in developing seeds (14) and root tips (19).

Seedling roots are fast-growing and are characterized by high metabolic activity. Roots of susceptible plant species appear to be very sensitive to cycloxydim and its related compounds (3, 13, 16, 17, 18, 19, 21, 22, 23). Root-tip culture has been suggested as suitable for studies of effects of compounds on root growth (37). Techniques for tissue-culturing root tips isolated from seedlings were described in 1943 (38). Hosaka and Takagi (21) first demonstrated the activity and selectivity of sethoxydim using excised root tips. Excised root tips in the same tissue culture system can grow continuously, develop lateral roots, respond with high sensitivity to cycloxydim, and develop the same reddening symptom as intact roots (see Chapter 4).

Hosaka and Takagi (21, 22) and Ishihara et al. (23) excised 0.2-cm tips from isolated roots cultured with sethoxydim and then analyzed the contents of various metabolites. Inhibition of lipids could be detected within 0.5 hours after treatment with 0.1  $\mu\text{M}$  sethoxydim. This sensitive technique will be used to examine metabolic effects and the mode of action of cycloxydim with the use of  $^{14}\text{C}$ -UL-glucose and -acetate. The long-term aim of this study is to elucidate the effect of cycloxydim on carbohydrate (glucose) metabolism and to locate the site of action and its relation to the reddening developed in sensitive root tissues.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Plant Material

Corn and soybean seeds were imbibed in water, surface-sterilized, and then germinated for 3 days. Details of the plant material preparation were described in Chapter 4. For the study of glucose metabolism, 1-cm root tips were excised and subsequently tissue cultured for 1 day prior to the cycloxydim treatments. During tissue culture, excised root tips grew uniformly to approximately 2 cm and showed no contamination. Therefore, the 1-day culturing period was eliminated from the acetate metabolism study; instead 1.5 cm root tips were excised from seedlings and used directly for treatment. The procedures for root tip tissue culture and cycloxydim treatments followed those of Hosaka and Takagi (22) with modifications described below.

### 6.2.2 Experiments on Metabolism of $^{14}\text{C}$ -Glucose or $^{14}\text{C}$ -Acetate in Root Tips Treated with Cycloxydim

Eleven root tips of corn and soybean were used for every replication in the experiment on  $^{14}\text{C}$ -glucose metabolism. Root tips were incubated in 2 ml of White's medium (38) containing 29.6 kBq  $^{14}\text{C}$ -UL-glucose (sp. act. 9.56 GBq/mmol) and 0, 1, or 10  $\mu\text{M}$  concentration of cycloxydim. To reduce radioactivity dilution in the  $^{14}\text{C}$ -acetate metabolism experiment, 1.5 ml of medium and only eight corn root tips or seven soybean root tips per replication were used. Fewer soybean root tips were used than corn root tips, because in the previous experiments soybean root tips took up and metabolized  $^{14}\text{C}$  precursors at a higher rate than did corn root tips. Root tips were incubated in 1.5 ml White's medium containing 55.5 kBq  $^{14}\text{C}$ -acetate (sp. act.  $3.6 \times 10^3$  kBq/mmol) and 0,

1, or 10  $\mu\text{M}$  concentration of cycloxydim. All treatments were replicated twice and the experiment was repeated at least once.

After 4 hours treatment, root tips were removed and rinsed with cold medium containing  $1 \times 10^{-3}\text{M}$  unlabeled glucose or acetate. Root tips incubated with  $^{14}\text{C}$ -glucose were sectioned at 0.2, 0.5, 1, and 1.5 cm, and these segments were used for analysis. Based on the results of the  $^{14}\text{C}$ -UL-glucose metabolism study, only the 2-mm root tips of the controls and the cycloxydim-treated seedlings showed differences in lipid synthesis. Therefore, in the experiment on  $^{14}\text{C}$ -acetate metabolism, only the 2-mm root tips were excised for analysis. For the identification of major carbohydrates in root tips, 40 root tips of 1 cm length were tissue cultured for 4 hours and then freeze dried for later analysis.

### **6.2.3 Tissue Extraction and Separation of Lipid- and Water-Soluble**

#### **Compounds**

Excised roots were placed in Eppendorf centrifuge tubes, chilled in ice, and hand macerated with a pestle. Tissues were extracted with 0.4 ml chloroform and methanol (2:1, v/v) at  $70^\circ\text{C}$  for 15 minutes. After cooling the tubes, root tissue was subsequently extracted with 0.45 ml chloroform:methanol:water (1:2:0.8, v/v/v) with constant shaking (200 cycles/minute) at  $25^\circ\text{C}$  for 1 hour. NaCl (0.15 ml of 1%) was added to the combined extracts. Chloroform and water phases were then partitioned after 5 minutes centrifugation in a clinical centrifuge. Radioactivity measurements were carried out using 100  $\mu\text{l}$  of each fraction mixed with 8 ml of scintillation fluid (Ecolume™ purchased from ICN Biomedicals, Inc., Irvine, CA 92713). For the identification of major carbohydrates, 40 root tips were extracted with the above procedure. Because of greater quantity of tissues, five times greater volume of each solvent was required.

## **6.2.4 Separation of Water-Soluble Compounds by Ion Exchange**

### **Chromatography**

Aliquots of the water layer were passed successively through columns containing AG® 50W-X8 (H<sup>+</sup>) cation-exchange resin, and AG® 1-X8 (formate) anion-exchange resin (BioRad Laboratories, Richmond, California 94804). Distilled water was applied to both columns for washing. Neutral compounds (sugars) passed through both columns. Compounds with positive charges (amino acids) bound to AG 50W-X8 were eluted with 4 N NH<sub>4</sub>OH. Negatively charged compounds (organic acids) bound to AG 1-X8 were eluted with 4 N formic acid. The eluates were dried under an air stream in a 45°C water bath. To quantify the radioactivity in these compounds, 0.2 ml of distilled water was added to dissolve the dried residues and then mixed with 8 ml of Ecolume for scintillation counting. For the identification of major carbohydrates in root tips, the dried residues were dissolved in 0.5 ml of 50% ethanol and then spotted on HPTLC plates.

## **6.2.5 HPTLC Analysis of Compounds Extracted from Root Tips**

Precoated high-performance thin-layer chromatography (HPTLC) plates, 10 × 10 cm, were purchased from CAMAG Scientific, Inc., Wrightsville Beach, NC 28480. Plates (HPTLC silica 60 Merck, without F) were used for lipid, amino acid, and sugar analyses. For the analysis of carboxylic acids, HPTLC cellulose plates, 10 × 10 cm, were used.

### 6.2.5.1 Lipid Analysis

Lipid standards were either purchased from Sigma Chemical Co., St. Louis, MO, 63178 or were generously supplied by Dr. D. M. Orcutt and Ms. Nina Hopkins in the Department of Plant Pathology, Physiology, and Weed Science, VPI&SU, Blacksburg, Virginia.

**Lipid standards:** Phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycine (PG), phosphatidylserine (PS), phosphatidylinositol (PI), linoleic acid (LIN), triglyceride (TRI), cholesterol (CHO), cholesteryl palmitate (CHOPAL), stearyl glucoside (SG), esterified stearyl glucoside (ESG), and lanosterol (LAN).

**Developing systems:** Two solvent systems were used.

1. Hexane:ethyl ether:glacial acetic acid (65:35:2 v/v/v)
2. Chloroform:methanol:water (85:15:0.5 v/v/v)

**Visualization:** Lipids on chromatograms were located by two ways.

1. by the development of brown color after dipping plates in a solution containing 3% copper acetate in 8% phosphoric acid and then charring the plates at 150°C for a few seconds. Results of the separation of lipid standards using solvent systems 1 and 2 are shown in Figure 6-1 and Figure 6-2, respectively.
2. by their fluorescence under ultraviolet light after spraying a fine mist of 1 mM TNS (2-p-toluidinylnaphthylene 6-sulfonate) (Sigma Chemical Co., St. Louis, MO, 63178) in 50 mM Tris buffer, pH 7.4.

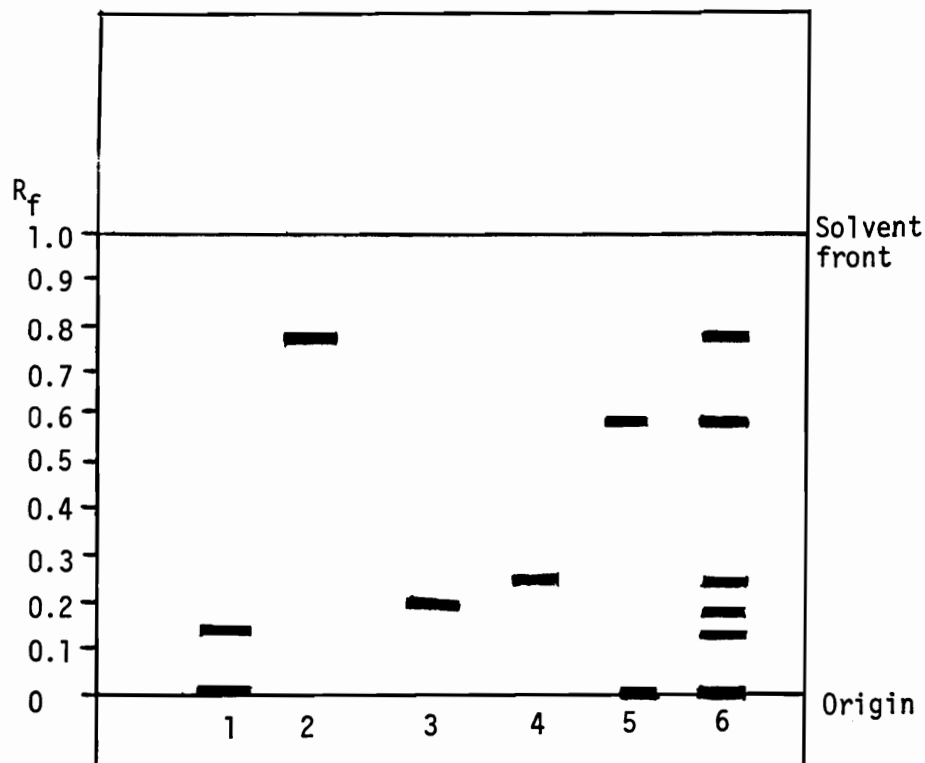
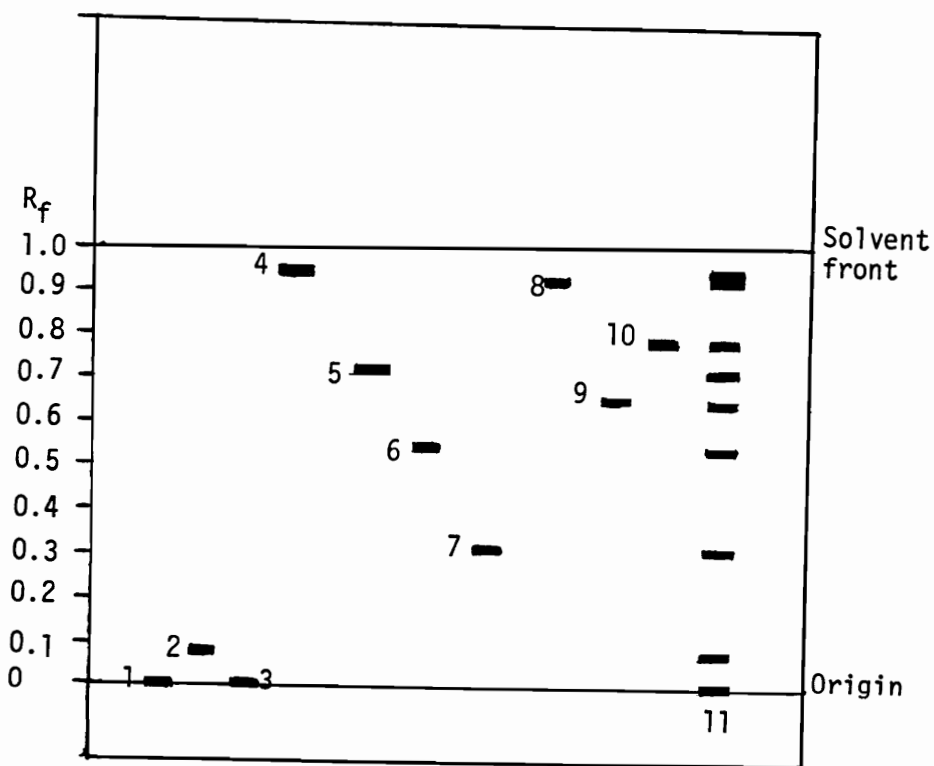


Figure 6-1. Separation of lipid standards using an HPTLC silica plate (10 cm × 10 cm) with solvent system 1 (hexane:ethyl ether:glacial acetic acid, 65:35:2 v/v/v): Compounds spotted on the individual lanes are: (1) a mixture of cholesterol, esterified stearyl glucoside, and stearyl glucoside; (2) cholesterol palmitate; (3) linoleic acid; SG; (4) lanosterol; (5) a mixture of phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine, and triglyceride; (6) a mixture mixture of all of the above compounds.



**Figure 6-2.** Separation of lipid standards using an HPTLC silica plate (10 cm × 10 cm) with solvent system 2 (chloroform:methanol:water, 85:15:0.5): Compounds spotted on the individual lanes are: (1) phosphatidylcholine; (2) phosphatidylethanolamine; (3) phosphatidylinositol; (4) triglyceride; (5) cholesterol; (6) esterified stearyl glucoside; (7) stearyl glucoside; (8) cholesteryl palmitate; (9) linoleic acid; (10) lanosterol; (11) a mixture of all the above compounds.



### 6.2.5.2 *Analysis of Carboxylic Acids*

Carboxylic acids were analyzed using the HPTLC procedure of Lin and Tanner (29).

**Standards:** Trans-aconitic acid, citric acid, malic acid,  $\alpha$ -keto-glutaric acid, and succinic acid.

**Solvent system:** Ethyl acetate:toluene:water:formic acid (60:20:20:15 v/v/v/v). This mixture was shaken vigorously and allowed to sit overnight in a refrigerator. After 12 hours, the upper clear layer was used as the developing solvent.

**Staining reagent:** Equal volumes of (1) and (2) were mixed and sprayed onto the plates.

1. 2 grams of xylose dissolved in 6 ml of water and then diluted to 100 ml with methanol.
2. 2 ml of aniline dissolved in 100 ml of methanol

### 6.2.5.3 *Analysis of Amino Acids*

**Solvent system:** Methylene chloride:methanol:ammonia (40:40 :20 v/v/v).

**Visualization:** Amino acids were visualized after spraying with 2% ninhydrin in ethanol.

**Standards:** Glycine, glutamine, leucine, isoleucine, asparagine, and phenylalanine.

#### 6.2.5.4 *Analysis of Sugars*

**Pretreatment of HPTLC plates:** Plates were pretreated with sprays of 0.1 M sodium bisulfite in a diluted citrate buffer (1 part buffer and 9 parts water) and then heated at 110°C for 1 hour. Samples were then spotted on plates.

**Solvent system:** The plate was developed three times in the same direction using acetonitrile:water (85:15).

**Visualization:** Plates were dipped in 0.1 N ceric sulfate in 2 N H<sub>2</sub>SO<sub>4</sub> and then charred at 110°C for 15 minutes.

**Standards:** Xylose, ribose, glucose, fructose, and sucrose.

#### 6.2.6 HPTLC Analysis of <sup>14</sup>C-Lipids in Roots

Aliquots of chloroform extracts of root tips after incubation with <sup>14</sup>C-acetate and lipid standards were spotted on an HPTLC silica plate (Camag). Plates were developed using chloroform:methanol:water (85:15:0.5 v/v). The plates were then sprayed with TNS staining solution and viewed under UV. Fluorescent bands that corresponding to the selective standard phospholipids and free sterols were scraped out, pressed into fine powder, and mixed with 8 ml Ecolume for assaying radioactivity. All of the <sup>14</sup>C determination were made on a Beckman LS-255 scintillation counter.

## 6.3 RESULTS AND DISCUSSION

### 6.3.1 Effect of Cycloxydim on $^{14}\text{C}$ Distribution and $^{14}\text{C}$ -Glucose

#### Metabolism in Corn and Soybean Root Tips

Cells and tissues in corn and soybean root tips (2-mm terminal end of roots) include the meristematic tissues. The adjoining root segments, 0.2 to 0.5 cm, 0.5 to 1 cm, and 1 to 1.5 cm from the root tip, are tissues of successively higher differentiation and maturation and lower metabolic activity. The total  $^{14}\text{C}$  radioactivity recovered from extracts of corn root segments increased as the lengths of the segment increased despite their different metabolic activities (Table 6-1). This is probably attributable to the greater amount of tissue available for absorption.

The total  $^{14}\text{C}$  recovered from extracts of root tip segments treated with 1  $\mu\text{M}$  cycloxydim was similar to that from the controls. Segments of root tips treated with 10  $\mu\text{M}$  cycloxydim showed reduced  $^{14}\text{C}$  radioactivity recovered in root segments behind the meristematic tip. Most of the  $^{14}\text{C}$  radioactivity was found in the aqueous extracts of both treated and untreated root tissues. Over 95% and 98% of the total  $^{14}\text{C}$  were recovered in the aqueous extracts of meristematic tissues (0 to 0.2 cm segments) and tissues (0.5 to 1.0 cm segments) with relatively low metabolic activity, respectively.

The lipids in root tips contained 5% or less of the total radioactivity recovered. The meristematic tips (0 to 0.2 cm segments) incorporated the most  $^{14}\text{C}$  into lipids among all root segments examined. Root tissues farther from the apex showed a successive decrease in labeled lipids. Cycloxydim reduced the incorporation of  $^{14}\text{C}$  into lipids in the apex in a dose-dependent fashion.

**Table 6-1. Radioactivity of water- and chloroform-soluble compounds extracted from untreated and cycloxydim-treated corn root segments 4 hours after incubation with <sup>14</sup>C-glucose.**

Root segment	Cycloxydim concentration	Phase		$\Sigma^{14}\text{C}$ recovered
		$\text{CH}_3\text{OH-H}_2\text{O}(\%)^\dagger$	$\text{CHCl}_3 (\%)^\dagger$	
	$\mu\text{M}$	(Bq / 11 tips) $\ddagger$		
0-0.2 cm	0	144.5 $\pm$ 6.8 (95.2)	7.3 $\pm$ 0.4 (4.8)	151.8 $\pm$ 7.2
	1	147.8 $\pm$ 6.7 (97.0)	4.5 $\pm$ 0.2 (3.0)	152.3 $\pm$ 6.9
	10	141.8 $\pm$ 14.4 (97.5)	3.6 $\pm$ 0.2 (2.5)	145.4 $\pm$ 14.6
0.2-0.5 cm	0	268.9 $\pm$ 13.5 (98.0)	5.4 $\pm$ 0.2 (2.0)	274.3 $\pm$ 13.7
	1	254.4 $\pm$ 10.7 (98.3)	4.4 $\pm$ 0.3 (1.7)	258.9 $\pm$ 10.9
	10	200.6 $\pm$ 12.6 (98.0)	4.0 $\pm$ 0.2 (2.0)	204.5 $\pm$ 12.8
0.5-1.0 cm	0	335.0 $\pm$ 6.0 (98.5)	5.2 $\pm$ 0.2 (1.5)	340.2 $\pm$ 6.2
	1	330.6 $\pm$ 33.7 (98.7)	4.4 $\pm$ 0.4 (1.3)	334.9 $\pm$ 34.1
	10	245.5 $\pm$ 30.9 (98.5)	3.7 $\pm$ 0.2 (1.5)	249.2 $\pm$ 30.8
1.0-1.5 cm	0	352.6 $\pm$ 16.2 (98.8)	4.3 $\pm$ 0.1 (1.2)	356.9 $\pm$ 16.3
	1	265.0 $\pm$ 41.3 (98.6)	3.6 $\pm$ 0.3 (1.4)	268.6 $\pm$ 41.6
	10	201.7 $\pm$ 26.2 (98.5)	3.2 $\pm$ 0.1 (1.5)	204.9 $\pm$ 26.3

$\dagger$  Numbers are the percent of total <sup>14</sup>C radioactivity recovered.

$\ddagger$  Means values  $\pm$  SD of four replicates from two experiments.

On the other hand, cycloxydim did not affect  $^{14}\text{C}$ -glucose uptake and metabolism in soybean root tips. The total  $^{14}\text{C}$  recovered and incorporated into water- and lipid-soluble compounds in untreated and treated soybean meristematic root tips were similar (Table 6-2).

The total  $^{14}\text{C}$  recovered in extracts of untreated and treated corn roots did not differ within the limits of one standard deviation. The incorporation of  $^{14}\text{C}$  from  $^{14}\text{C}$ -glucose into water-soluble compounds in 2-mm root apex of both treated and untreated roots were similar. However, a reduction of  $^{14}\text{C}$  labelling into water-soluble compounds in cycloxydim-treated root segments behind the root apex was observed. The aqueous extract of root segments treated with 10  $\mu\text{M}$  cycloxydim contained the lowest radioactivity. Radioactivity of these treated roots was of relatively high degree of variability. In order to characterize the radioactive compounds in the water-soluble extracts of untreated and cycloxydim-treated corn roots, aliquots of the extracts were fractionated on ion exchange columns into sugars, amino acids, and carboxylic acids. Approximately 75%, 15%, and 8% of the  $^{14}\text{C}$  activity recovered from 2-mm tips were represented by sugars, amino acids, and carboxylic acids, respectively (Table 6-3). Similarly, 81%, 4%, and 6% of the  $^{14}\text{C}$  activity recovered from 0.5 to 1 cm segments were found in sugars, amino acids, and carboxylic acids, respectively (Table 6-3). Most of the radioactivity in all water-soluble extracts was found in the sugar fractions. A dominant component of the sugar fraction was later determined to be glucose (see section 6.3.3). Meristematic tissues appeared to metabolize more of the  $^{14}\text{C}$ -glucose resulting in the production of more amino acids and carboxylic acids than in root tissues located at 0.5 to 1 cm from the tips.

### **6.3.2 Effects of Cycloxydim on $^{14}\text{C}$ Distribution and $^{14}\text{C}$ -Acetate**

#### **Metabolism in Corn and Soybean Root Tips**

Due to the sensitivity of lipid synthesis in the 1-mm terminal root tips to cycloxydim observed in the  $^{14}\text{C}$ -glucose metabolism study, the effect of cycloxydim on  $^{14}\text{C}$ -acetate metabolism was examined. The  $^{14}\text{C}$  recovered from the aqueous fractions and recovered in total from tissues of

**Table 6-2. Radioactivity of water- and chloroform soluble compounds extracted from untreated and cycloxydim-treated soybean root tips (the 2-mm root apices) 4 hours after incubation with  $^{14}\text{C}$ -glucose.**

Cycloxydim concentration	Phase		$\Sigma^{14}\text{C}$ recovered
	$\text{CH}_3\text{OH}\cdot\text{H}_2\text{O}$	$\text{CHCl}_3$	
$\mu\text{M}$	(Bq / 11 root tips)†		
0	$157.8 \pm 5.4$	$9.1 \pm 0.8$	$166.9 \pm 6.2$
10	$159.4 \pm 5.1$	$8.9 \pm 0.6$	$168.3 \pm 4.5$

† Numbers are means  $\pm$  SD of four replicates.

**Table 6-3. Radioactivity of sugars, amino acids, and organic acids extracted from untreated and cycloxydim-treated corn tips 4 hours after incubation with <sup>14</sup>C-glucose.**

Root segment	Fraction	Cycloxydim rate					
		0	(%)	1 $\mu$ M	(%)	10 $\mu$ M	(%)
(Bq / 11 root tips) <sup>†</sup>							
0-0.2 cm	Sugars	56.7 $\pm$ 1.9	(76.4)	54.7 $\pm$ 2.5	(75.4)	52.8 $\pm$ 4.9	(74.6)
	Amino acids	11.8 $\pm$ 1.2	(16.0)	11.6 $\pm$ 0.7	(16.4)	11.6 $\pm$ 0.9	(16.4)
	Carboxylic acids	5.6 $\pm$ 0.5	(7.6)	6.2 $\pm$ 0.7	(8.4)	6.4 $\pm$ 0.7	(9.0)
	Total <sup>‡</sup>	74.1 $\pm$ 3.6	(100.0)	72.4 $\pm$ 3.9	(100.0)	70.8 $\pm$ 6.5	(100.0)
0.5-1 cm	Sugars	148.1 $\pm$ 10.5	(89.2)	143.5 $\pm$ 12.9	(89.0)	—	—
	Amino acids	7.4 $\pm$ 0.7	(4.4)	7.2 $\pm$ 0.9	(4.5)	—	—
	Carboxylic acids	10.8 $\pm$ 0.7	(6.4)	10.5 $\pm$ 1.2	(6.5)	—	—
	Total <sup>‡</sup>	166.3 $\pm$ 11.9	(100.0)	161.2 $\pm$ 15.1	(100.0)	—	—

<sup>†</sup> Numbers are means  $\pm$  SD of four replicates from two experiments.

<sup>‡</sup> One half of the CH<sub>3</sub>OH-H<sub>2</sub>O-phase in Table 6-1 were fractionated on the ion exchangers AG<sup>®</sup> 50W-X8 and 1-X8.

control and treated corn roots were similar, though with some variation (Table 6-4). The variability tended to increase in roots treated with increased concentrations of cycloxydim. The distribution of  $^{14}\text{C}$  from  $^{14}\text{C}$ -acetate into chloroform- and water-soluble compounds was relatively even, with slightly more  $^{14}\text{C}$  being distributed into water-soluble compounds. Cycloxydim reduced the  $^{14}\text{C}$  incorporation into lipids in corn root tips in a dose-dependent fashion. With increasing concentration of cycloxydim, there appeared to be an increase of radioactivity in water-soluble compounds.

The aqueous extract containing the highest  $^{14}\text{C}$  activity was further fractionated on ion exchange columns. The fractionated eluates containing sugars, amino acids, and organic acids all showed high  $^{14}\text{C}$  activity (Table 6-5). When presented as ratios based on the total radioactivity recovered, the  $^{14}\text{C}$  from  $^{14}\text{C}$ -acetate distributed in the water-soluble compounds similarly to that of the control. Results suggested that these high levels of  $^{14}\text{C}$  in the aqueous extract of root tissues treated with  $10\ \mu\text{M}$  cycloxydim were due to the high incorporation and metabolism of  $^{14}\text{C}$ -acetate.

Lipids such as phosphatidylethanolamine (PE), representing the lipid class containing fatty acids, and sterols (ST) and sterol glycosides (SG), representing lipids without a fatty acid moiety, were separated by HPTLC and quantified. As shown in Table 6-6, the  $^{14}\text{C}$  incorporation into PE was reduced, and  $^{14}\text{C}$  incorporation into ST and SG in root tips treated with 1 and  $10\ \mu\text{M}$  cycloxydim was slightly affected.

In contrast to corn root tissues, lipids in soybean root tips were not affected by cycloxydim. As shown in Table 6-4, soybean root tips treated with  $10\ \mu\text{M}$  cycloxydim showed similar total amounts of  $^{14}\text{C}$  recovered and  $^{14}\text{C}$  distribution into water- and chloroform-soluble compounds.

### **6.3.3 Identification of the Major Water-Soluble Metabolites in Corn Root Tips**

Based on the HPTLC analysis, corn root tips 1 cm long contain a relatively high amount of sucrose, glucose, and fructose in the sugar fraction (Figure 6-3). There appeared to be one domi-



**Table 6-4. Radioactivity of water- and chloroform-soluble compounds recovered from untreated and cycloxydim-treated corn and soybean root tips incubated with <sup>14</sup>C-acetate.**

Species	Cycloxydim concentration	Phase				$\Sigma^{14}\text{C}$ recovered
		CH <sub>3</sub> OH-H <sub>2</sub> O	(%)†	CHCl <sub>3</sub>	(%)†	
	$\mu\text{M}$	$(\times 10^2 \text{ Bq} / 8 \text{ root tips})\ddagger$				
Corn	0	15.6 ± 0.7	(51)	14.9 ± 0.2	(49)	30.6 ± 0.9
	1	15.3 ± 1.9	(57)	11.3 ± 0.4	(43)	26.6 ± 2.3
	10	19.5 ± 5.2	(66)	9.9 ± 0.7	(34)	29.4 ± 5.9
		$(\times 10^2 \text{ Bq} / 7 \text{ root tips})\ddagger$				
Soybean	0	17.1 ± 0.5	(53)	15.3 ± 1.0	(47)	32.4 ± 1.5
	10	16.6 ± 1.1	(51)	16.2 ± 0.6	(49)	32.8 ± 1.7

† % of the total <sup>14</sup>C recovered.

‡ Numbers are the means ± SD of four replicates.

**Table 6-5. Radioactivity in water-soluble compounds from controls and cycloxydim-treated corn root tips incubated with <sup>14</sup>C-acetate 4 hours after treatment.**

Fraction	Control	(%)‡	10μM	(%)‡
(Bq / 8 root tips)				
Sugars	7.5	(3.8)	9.0	(3.1)
Amino acids	109.0	(55.1)	156.7	(54.9)
Carboxylic acids	81.3	(41.1)	119.7	(42.0)
Total	197.8	(100.0)	285.4	(100.0)
Radioactivity applied in the ion exchanger	240.0		351.6	

† 100 μl of 650 μl water-soluble phase in Table 6-4 was fractionated on the ion exchangers AG® 50W-X8 and 1-X8. Samples analyzed included one of the controls and one of the 1 μM treated replicates which contained the highest radioactivity.

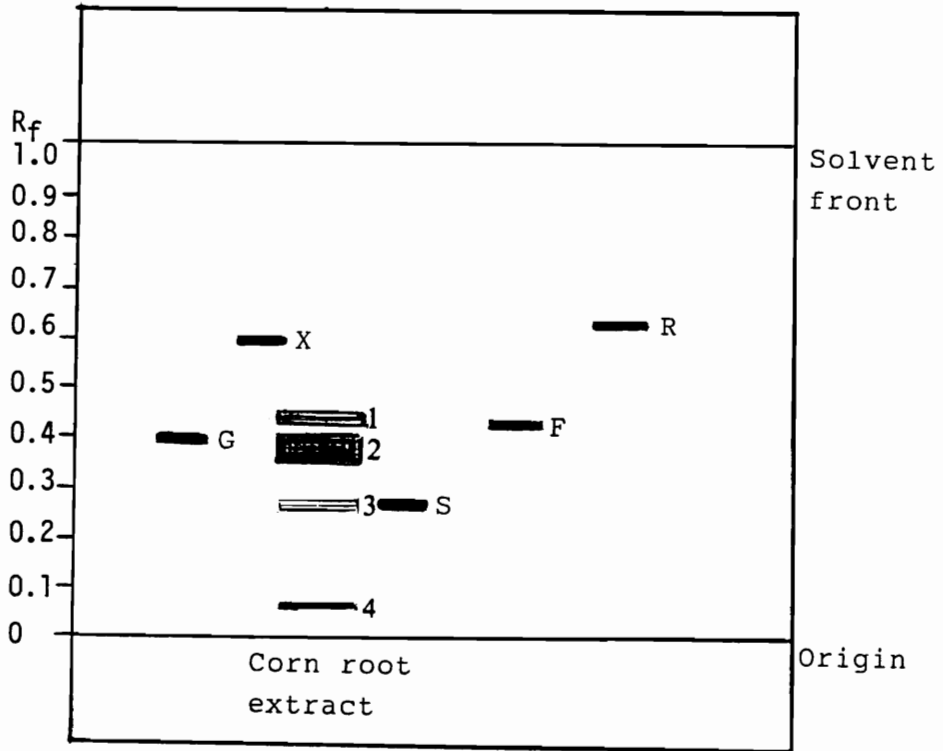
‡ % of the total <sup>14</sup>C recovered from column chromatography.

**Table 6-6. HPTLC results of <sup>14</sup>C (dpm) in selected fatty acid-containing and non-fatty acid-containing lipids extracted from controls and cycloxydim-treated root tips incubated with <sup>14</sup>C-acetate for 4 hours.**

Selected lipids‡	Cycloxydim rate			
	0	1 μM (%)†	10 μM (%)†	
	(Bq / 8 root tips)			
Lipid containing fatty acids				
PE	32.7	24.5 (75)	19.2 (58)	
Lipids containing no fatty acid				
ST	45.8	47.2 (103)	41.3 (91)	
Radioactivity spotted on HPTLC	250.0	183.3 (73)	160.1 (64)	

† % of untreated.

‡ PE, Phosphatidylethanolamine; ST, Sterols; SG, Sterol glycosides.  
Lipids were separated by HPTLC using the solvents:  
chloroform:methanol:water (85:15:0.5 v/v/v).



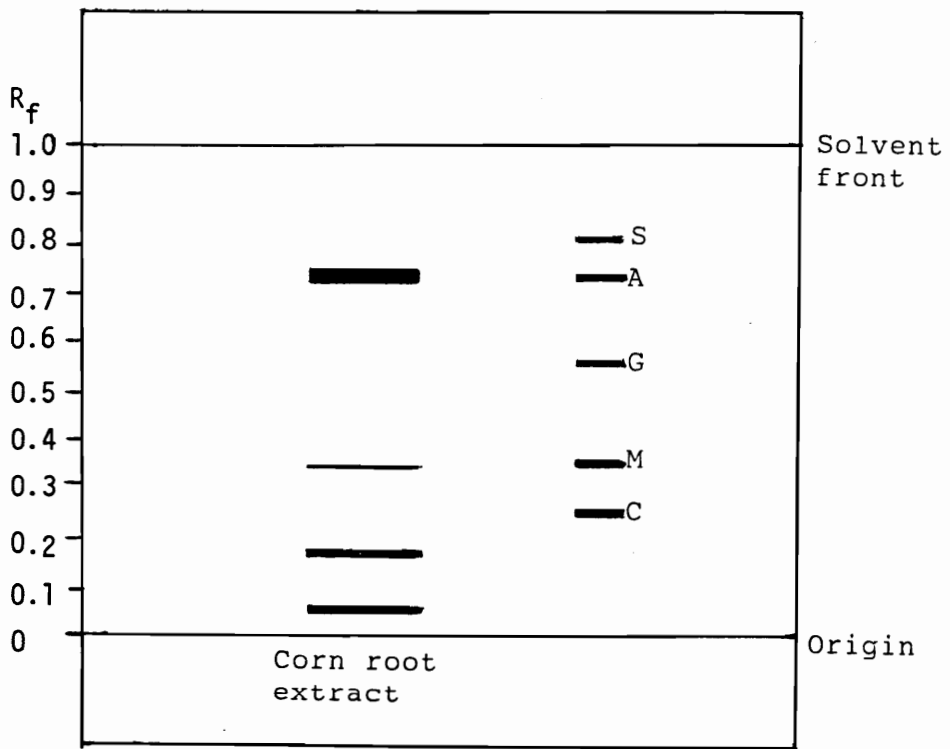
**Figure 6-3.** HPTLC analysis of sugars extracted from corn root tips.: Four bands (1 through 4) of corn root extract (the sugar fraction) as well as bands of standards showed on an HPTLC silica plate (10 cm × 10 cm) after development and visualization spray as described in the Materials and Methods. G, glucose; X, xylose; S, sucrose; f, fructose; R, ribose.

nant acid present in the organic acid fraction of the root tips extract (Figure 6-4). This acid was identified as aconitate based on the similar Rf value as that of the standard. This acid was reported to be the dominant acid present in corn leaf extracts (20). Corn root tips contained a number of amino acids that reacted with ninhydrin (data not shown). One-way HPTLC analysis did not provide enough resolution to separate out most of the amino acids in the extracts. Results demonstrate that fractionation of water-soluble compounds by the ion exchangers are effective in separating sugars, carboxylic acids, and amino acids.

### 6.3.4 Conclusion

Cycloxydim reduced the incorporation of  $^{14}\text{C}$  into lipids in corn root tips consistently with either  $^{14}\text{C}$ -glucose or -acetate used as the precursor. Based on lipid synthesis, meristematic tissues appear to be most sensitive to cycloxydim among all root tissues examined. Corn root tips treated with  $10\ \mu\text{M}$  cycloxydim and incubated with  $^{14}\text{C}$ -glucose showed reduced incorporation of  $^{14}\text{C}$  into lipids. This was perhaps due to the low uptake of the precursor, as evidenced by the low  $^{14}\text{C}$  present in the water-soluble fraction and total  $^{14}\text{C}$  recovered from the extracts. Because more of the  $^{14}\text{C}$  from  $^{14}\text{C}$ -acetate was incorporated into lipids, lipids in root tissues treated with 1 and  $10\ \mu\text{M}$  cycloxydim and incubated in this precursor were further separated by HPTLC and quantified. Incorporation of lipids containing a fatty acid moiety, such as PE, was inhibited by cycloxydim; whereas, the incorporation of lipids containing no fatty acid such as sterols and sterol glycosides was slightly affected. The inhibitory effect of cycloxydim on lipid synthesis and specifically on the synthesis of fatty acid-containing lipids in the corn root system is similar to that of sethoxydim and some PCAs in leaves and shoots reported in the literature (4, 8, 11, 15, 18, 19, 20, 21, 22, 23, 26, 27).

In the experiment with root tips and  $^{14}\text{C}$ -acetate, some replicates treated with  $10\ \mu\text{M}$  cycloxydim showed reduced labeled lipids and contained relatively high amounts of water-soluble



**Figure 6-4.** HPTLC analysis of carboxylic acids extracted from corn root tips.: One distinct band of corn root extract (the carboxylic acid fraction) and bands of standards showed on a HPTLC cellulose plate (10 cm  $\times$  10 cm) after development and visualization spray as described in the Materials and Methods. A, trans-aconitic acid; S, succinic acid; G, glycolic acid; M, malic acid; C, citric acid.

$^{14}\text{C}$  metabolites in the tissue extracts when compared to controls. Although the inhibitory effect of cycloxydim on lipid synthesis is apparent, its impact on the  $^{14}\text{C}$  flow into water-soluble compounds cannot be determined based on the high degree of variation in the data. Similar variability was reported by Hoppe (20) using either leaf discs or isolated root tips 1 cm long in similar experiments designed to study the effect of diclofop-methyl on metabolism of  $^{14}\text{C}$ -acetate. In an experiment with detached leaves that were treated with diclofop-methyl and then incubated with  $^{14}\text{C}$ -acetate, Hoppe (20) found a reduction of  $^{14}\text{C}$  incorporated into lipids coincident with an apparent increase of  $^{14}\text{C}$  flow into water-soluble compounds. This distinct trend of distribution of  $^{14}\text{C}$  was not observed consistently in other experiments. Hoppe attributed this increased flow of  $^{14}\text{C}$  into water-soluble compounds in detached and intact leaves to the higher uptake of the labeled precursor via the transpiration stream. In addition, there are factors that may cause this different  $^{14}\text{C}$  flow into water-soluble and chloroform-soluble compounds in these experiments.

The herbicidal treatments in experiments for Hoppe's plant materials were different. In his experiment, the leaves were detached from plants foliarly pretreated with diclofop-methyl for different time periods (0.5 hr, 2 hr, and 4 hr) and then placed in a herbicide-free solution of labeled precursor for incubation. In other experiments, root tips and leaf discs were treated directly with herbicide and  $^{14}\text{C}$  precursor. The subsequent incubation of detached leaves from treated plants may dilute diclofop-methyl present in the leaves. During this incubation period, leaf tissues may resume or accelerate metabolic activities that were not affected by the herbicide to repair injured tissues by synthesizing compounds rapidly with the use of the suddenly available acetate. With this, I agree with Hoppe's statement (20) "the increase of  $^{14}\text{C}$  flow into water-soluble compounds may not be a direct effect of diclofop-methyl but a consequence of the inhibition of fatty acid biosynthesis".

The experiments with the use of excised root tips for studying the effects of cycloxydim and its related compounds are plagued by variation in the data. Several factors potentially can cause such variation. First, the dissection of the 0.2 cm terminal ends of root tips after treatments may cause the loss of labeled compounds through the cut surface, especially the loss of water-soluble compounds. Secondly, not all the root tissues in the root tips received a uniform treatment of

cycloxydim. The gradient effect of cycloxydim in root tips demonstrated in the morphological study may contribute much to the variation in data. Thirdly, this gradient effect of cycloxydim caused the greater injury in the outer layers of the root tissues, which may allow leakage of labeled compounds, especially the water-soluble compounds, through the wounded cells during the incubation and at the post-treatment rinse. The leakage of amino acids in root tips treated with diclofop-methyl was documented by Hoppe (17). Lipids, being water-insoluble, may remain in cells with less disturbance and therefore be quantified with relative consistency.

Compared to approximately 5% of the total  $^{14}\text{C}$  incorporated into lipids in root tips treated with  $^{14}\text{C}$ -glucose, one half of the total  $^{14}\text{C}$  recovered was incorporated into lipids in roots treated with  $^{14}\text{C}$ -acetate. Acetate is apparently a preferred precursor over glucose for fatty acid synthesis in root tips. With  $^{14}\text{C}$ -glucose as the precursor in experiments of root tips treated with cycloxydim, the lack of apparent alteration in the  $^{14}\text{C}$  flow into water-soluble compounds in these root tips may be due to the fact that the impact caused by the inhibition of lipid synthesis was too small during such a relatively short period of incubation. Root tissues were slow in breaking down sugar molecules as shown by most of the radioactivity retained in the sugar fraction and relatively small portion of the  $^{14}\text{C}$  activity found in the amino acid and carboxylic acid fractions. Root tips appear to have aconitate as a dominant acid. The accumulation of this acid may further slow the distribution flow of  $^{14}\text{C}$  from labeled precursors into compounds involved in the TCA cycle and its associated metabolic pathways.

Gurr and his colleagues (14) used  $^{14}\text{C}$ -glucose and  $^{14}\text{C}$ -acetate as precursors to study the synthesis of lipids in developing seeds. They reported that with the use of  $^{14}\text{C}$ -UL-glucose all radioactivity was located in glycerol moiety of lipids, whereas with  $^{14}\text{C}$ -acetate used as precursor 97% of the incorporated radioactivity was recovered in the fatty acid moiety. Plant tissues do not use acetate by directly breaking down glucose molecules through glycolysis for the synthesis of fatty acids. Only the glycerol moiety of glucose and the glucose molecule as a whole were used directly for lipid synthesis. Based on this information, the reduction of incorporation of  $^{14}\text{C}$  from  $^{14}\text{C}$ -glucose may be an indirect effect of cycloxydim and a consequence of its fatty acid inhibition in the treated roots.



Although the system with excised root tips appeared to be sensitive to cycloxydim in bioassays, the definitive results of effects of cycloxydim especially on  $^{14}\text{C}$ -UL-glucose metabolism were not obtainable due to the high degree of variation. However, the results demonstrate that sugars are the major carbohydrates in roots and are metabolized slowly. The color change of susceptible plants developed at the later stage of cycloxydim treatments may be due to conversion of sugars into anthocyanin. Anthocyanin synthesis may not be the direct effect of cycloxydim but an induced mechanism for defense or a senescence process responding to cycloxydim injury.

The reduction in the size of root tips (2 mm) used as experimental material compared to that (1 cm) used by Hoppe did not improve the variation problem, although the inhibition of lipid synthesis was consistent. Ishihara and his colleagues (23) and Hosaka and Takagi (21, 22) used this system and reported the effect of sethoxydim on the incorporation of several precursors into their respective metabolites with clear results. The use of this technique for determining the metabolism of a precursor with such a wide scope as used in this paper should be limited in the future due to the possible involvement of several factors potentially causing the data variation as discussed.

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## **7.0 EFFECTS OF CYCLOXYDIM ON ACETYL COENZYME A CARBOXYLASE IN CORN AND SOYBEAN ROOT TISSUES**

### ***7.1 INTRODUCTION***

Acetyl Coenzyme A carboxylase (ACCase) (acetyl-Coenzyme A: bicarbonate [EC 6.4.1.2]), which catalyzes carboxylation of acetyl-CoA to malonyl CoA, is the first committed enzyme in the biosynthesis of fatty acids. ACCase has been isolated and studied in microorganisms, animal tissues, and higher plants (2, 10, 11, 13, 15, 16, 28, 29, 32, 33, 35, 41, 42). This enzyme is composed of at least three proteins including a biotin carboxylase, a carboxyltransferase, and a biotin carboxyl carrier protein (BCCP). BCCP plays a key role in facilitating the two enzyme activities of ACCase. A high homology found in the DNA sequence of BCCP from many sources (16) indicates that BCCP is a well-conserved protein during evolution.

In higher plants, ACCase has been found in various tissues and cells, including developing seeds, embryonic tissues, cell culture, and leaf tissues (4, 5, 8, 9, 28, 29, 34, 35, 36, 37, 38, 39, 40, 41). The activity of ACCase in plants associated with fatty acid synthesis is known to be localized in plastids, the cellular compartments active in fatty acid biosynthesis. In addition, the presence of a cytosolic ACCase in developing jojoba (*Simmondsia chinensis*) seeds was suggested (41).

In green leaves, the chloroplast ACCase was inhibited by representative compounds of cyclohexen-ones and polycyclic alkanolic acids (PCAs) in a dose-dependent fashion (4, 5, 9, 34, 36, 37, 38, 39, 40, 44). The inhibition of this enzyme correlates with the inhibition of lipid synthesis by these herbicides (17, 30, 31). ACCase is also found in etiolated plant tissues (40). ACCase isolated from etiolated shoots of tolerant and susceptible plants showed similar sensitivity to these herbicides as did ACCase isolated from green leaf tissues and shoots.

Plant roots, although not the application target of cyclohexen-ones and PCAs, have shown similar sensitivity and developed similar injury symptoms as do leaves and whole plants when treated with these herbicides (3, 14, 18, 19, 20, 21, 22, 23, 24, 25, 27). Cycloxydim inhibits the elongation of intact corn roots and excised corn root tips in tissue culture (Chapters 3 and 4). The incorporation of  $^{14}\text{C}$ -UL-glucose and -acetate into lipids in root meristems was inhibited within a short time by a relatively low concentration of cycloxydim (Chapter 5). In order to investigate further the mechanism of lipid inhibition by cycloxydim in root systems, ACCase was isolated from root tissues of tolerant and susceptible plants and the activity of ACCase examined in the presence of cycloxydim.

## **7.2 MATERIAL AND METHODS**

### **7.2.1 Chemicals**

Acetyl-CoA, ATP, DTT (dithiothreitol), and  $\text{NaH}^{14}\text{CO}_3$  were purchased from Sigma Chemical Co., St. Louis, MO 63178. BCA Protein Assay Reagents A and B were purchased from Pierce, Rockford, Illinois 61105. Sethoxydim and cycloxydim, both technical grade and formulated, were supplied by BASF company. Technical grade sethoxydim was a lithium salt, containing 75% active ingredient. Technical cycloxydim contained 65% active ingredient.

### **7.2.2 Growing Plant Materials**

Corn kernels and soybean seeds were immersed in distilled water for 24 hours. Seeds were then surface sterilized with 33% commercial bleach solution for 15 minutes. Thereafter all utensils used in this experiment were sterilized at 120 °C and 103.4 kPa for 20 minutes. All procedures were carried out under aseptic conditions. Seeds were placed in trays with germination papers moistened with distilled water. Germination was allowed to proceed in an incubator with 24°C in the dark for five days.

### **7.2.3 Extraction and Partial Purification of ACCase**

The methods of Secor and Cséke (38) were followed. All procedures were carried out at 4°C in a cold room. Root tissues of corn and soybean were excised from corn and soybean seedlings.

Corn root tissue and soybean roots were ground in a mortar and pestle in a medium of 100 mM Tricine-KOH pH 8.3, 10% [v/v] glycerol, 10 mM b-mercaptoethanol, 1 mM Na<sub>2</sub>EDTA, and 1 mM phenylmethyl sulfonyl fluoride, and washed sand (tissue:medium, 2:1, w:v). The extracts were filtered through four layers of cheese cloth and centrifuged at 30,000 g for 20 minutes. The supernatant was collected. Polyethylene glycol (Sigma P-2139) was added to the supernatant to make a 6% (w/v) solution. After being stirred for 20 minutes, the solution was centrifuged at 30,000 g for 20 minutes. The supernatant was made to 14% polyethylene glycol and stirred for 20 minutes. The resulting pellet was saved and suspended with 10 mM Tricine-KOH, pH 7.8, and 10% glycerol. This solution was centrifuged at 10,000 g for 5 minutes. The supernatant was used for enzyme assays.

#### **7.2.4 Protein Determination**

Protein was determined by reacting with BCA reagent using bovine serum albumin as a standard.

#### **7.2.5 Acetyl-CoA Carboxylase Assay**

Incorporation of NaH<sup>14</sup>CO<sub>3</sub> into acetyl-CoA to form malonyl-CoA via the enzyme acetyl-CoA carboxylase was assayed. The assay medium contained Tricine-KOH, pH 8.3, 5 mM MgCl<sub>2</sub>, 2mM DTT, 2 mM ATP, 10 mM NaH<sup>14</sup>CO<sub>3</sub> (18.5 × 10<sup>3</sup> kBq/mmol), 0.3 mM acetyl-CoA, various concentrations of herbicide treatments, and approximately 0.3 μg/μl protein. The total assay volume was 0.2 ml. The assay medium was placed in a 20-ml scintillation vial and incubated for 15 minute in a 35°C water bath before initiating the reaction with acetyl-CoA. All acetyl-CoA assays were conducted in a fume hood. After 20 minutes the reaction was stopped by adding 30



$\mu\text{l}$  of 12 N HCl. The contents in vials were heated in a 90°C sand bath and dried under a stream of air. The dried residue was dissolved in 250  $\mu\text{l}$  of distilled water and 10 ml of scintillation fluid. Radioactivity was quantified by liquid scintillation spectrometry.

## 7.3 RESULTS

### 7.3.1 Characteristics of ACCase Activity

Root extracts of corn and soybean seedlings showed ACCase activity by incorporating the  $H^{14}CO_3^-$  into acid- and heat-stable products with the use of this radiochemical assay. The activity is ATP-dependent and requires the presence of acetyl CoA (Table 7-1). ACCase activity in the corn root assay system increased with incubation time. (Figure 7-1).

### 7.3.2 Inhibition of ACCase Activity

ACCase activity from corn root was inhibited by approximately 30%, 47%, and 75% in the presence of cycloxydim at concentrations of 1, 10, 100  $\mu M$ , respectively, 15 minutes after treatment (Figure 7-2). Sethoxydim at the same concentrations inhibited corn root ACCase activity by 47%, 61%, and 90%, respectively. ACCase extracted from soybean roots was not affected by 100  $\mu M$  cycloxydim. Soybean ACCase activity was not affected by 10  $\mu M$  sethoxydim but was inhibited 20% by 100  $\mu M$  sethoxydim.

## 7.4 Discussion

Cycloxydim and sethoxydim showed similar selectivity and activity to ACCase from root systems of corn and soybean seedlings. Cycloxydim appeared to be less potent than sethoxydim. The

**Table 7-1. Substrate and cofactor requirements for corn root ACCase assay. Assay time was 15 minutes.**

Factors	ACCase activity (nmol HCO <sub>3</sub> <sup>-</sup> incorporated / mg protein)
No ATP	4.9
No acetyl CoA	3.3
No enzyme	n.d.†
Corn extract	96.2

† n.d.: not detectable.

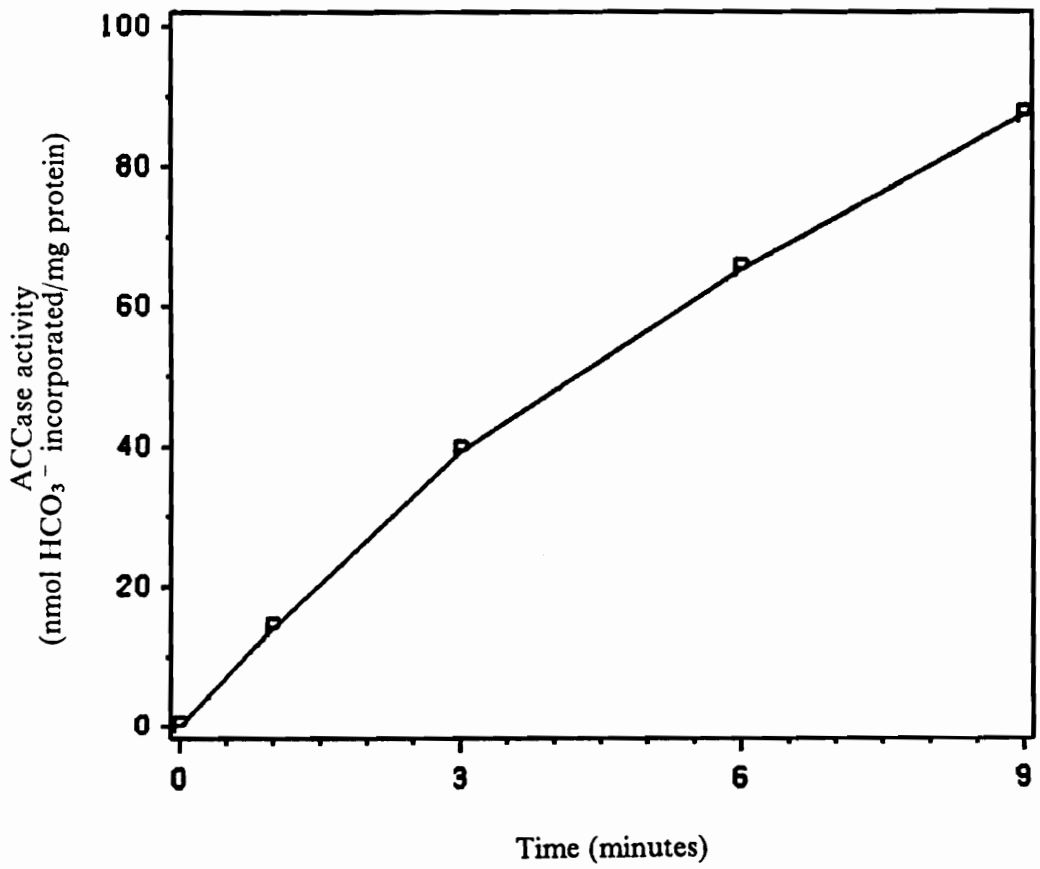


Figure 7-1. Time course of activity of ACCase isolated from untreated corn roots.

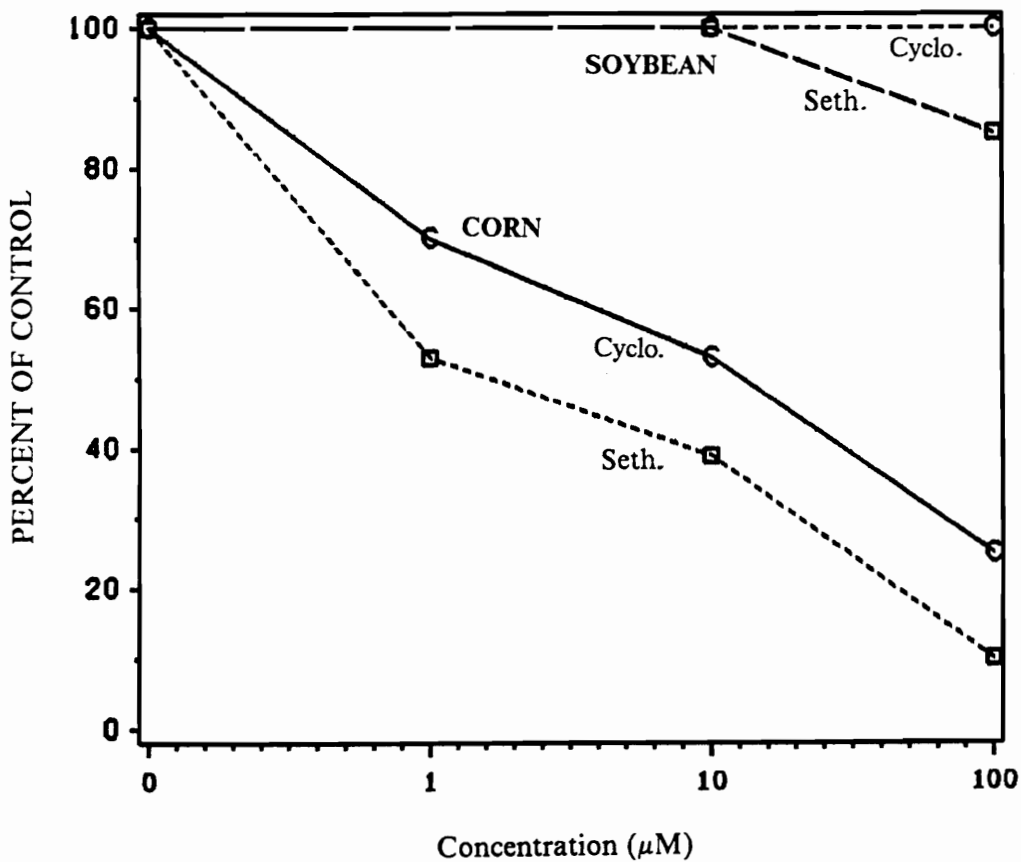


Figure 7-2. Dose response of cycloxydim (Cyclo.) and sethoxydim (Seth.) on activity of ACCase isolated from corn and soybean roots. Reaction time was 15 minutes.: Control rates for corn and soybean were 6.4 and 8.7 nmol  $\text{HCO}_3^-$  incorporated/min/mg protein, respectively.

relatively weaker activity of cycloxydim observed similarly in bioassays may be due to some degradation during prolonged storage. The selectivity and the dose-dependent inhibitory effects of cycloxydim and sethoxydim on ACCase in root systems are similar to those of ACCase extracted from other plant materials, such as isolated chloroplasts, green leaves, and etiolated shoots reported by other workers (4, 5, 9, 34, 36, 37, 38, 39, 40, 44). The sensitivity of this enzyme to cycloxydim also correlates well with the sensitivity of lipid synthesis in roots of susceptible corn plants (Chapter 6).

Rendina and his colleagues (36) recently reported that the carboxyltransferase site rather than the biotin carboxylase site of ACCase isolated from leaves was inhibited by representative compounds of cyclohexen-ones and PCAs. They suggested that these compounds may bind to the same region of this enzyme in leaves despite their different chemical structures. More work is needed to elucidate the specific ACCase binding site in root ACCase with the use of these compounds and demonstrate the inhibition mechanism of ACCase by these compounds in roots. Furthermore, ACCase activity assayed in this experiment was with a partially purified root homogenate. ACCase in roots may be localized in proplastids and/or cytoplasm. More research is necessary to isolate, separate, purify, and characterize the specific ACCase in plant roots and to determine their sensitivity to these herbicides.

Root systems of soybean and corn including intact roots, excised root tips, and root ACCase, showed consistent tolerance and susceptibility, respectively, to cycloxydim and sethoxydim. Roots appear to represent an effective system for examining the mode of action of herbicides of the cyclohexen-one and PCA classes. However the elucidation of the perhaps differential metabolism of cyclohexen-ones and PCAs in roots of susceptible and tolerant plants requires investigation in order to determine the basis (bases) of selectivity of these compounds in roots.

The differential metabolism of sethoxydim in tolerant and susceptible plants has been suggested as a possible basis of selectivity. Swisher and Corbin (43) reported that cell culture of soybean as well as tolerant soybean plants metabolized <sup>14</sup>C-sethoxydim at a faster rate than those of johnsongrass. However, Hatzios (17) reported that the lipid synthesis in isolated leaf cells of soybean was sensitive to sethoxydim treatment. Based on his results and a report by Campbell and

Penner (7) on indifferent absorption and translocation of sethoxydim between tolerant and susceptible plant species, Hatzios suggested that sethoxydim might possibly be rapidly metabolized in soybean plants to less phytotoxic transformation metabolites. These metabolites might be responsible for the observed tolerance of soybean in the field.

Campbell and Penner (7) reported sethoxydim to be metabolized rapidly in plants. The detailed structures of many of the transformation metabolites were not identified. Light degradation of sethoxydim also complicates (6) the study on the metabolism of this compound in plants. Recently the metabolism of cycloxydim in soybean has been reported (26). The metabolism of cycloxydim in soybean was reported to be rapid and very complex. Although many transformation products of cycloxydim were identified, the stability and phytotoxicity of these compounds were not reported. At present no information is available on the metabolism of cycloxydim in susceptible plants to compare with that in tolerant plants.

According to other reports, lipid synthesis and ACCase activity in isolated chloroplasts of mung bean (*Phaseolus aureus*) (37) and pea (*Pisum sativum* L., 'PI 9901-C') leaves (5), species closely related to soybean, were unaffected by sethoxydim and some PCA compounds, which is consistent with the observed tolerance of whole plants to these herbicides. ACCase from soybean root tissues examined in this paper showed much less sensitivity than that of corn. Further research is needed to explain the apparent differences in tolerance shown by soybean with the related compounds sethoxydim and cycloxydim at the whole plant, explant, and root extract levels to the susceptibility shown at the isolated leaf cell level. This could be accomplished by examining the ACCase from these plant materials along with metabolism studies of these herbicides.

The spectrum of plant species that are controlled selectively by cyclohexen-ones and PCAs deserves attention from a taxonomical point of review. Procaryotes and dicotyledonous species are known to be tolerant to these compounds, whereas most species in Poaceae, a monocotyledonous family, are susceptible. Some Poaceae species were reported to have a certain degree of tolerance to these compounds. As a closely related family to Poaceae, the Cyperaceae (the sedge family) is tolerant to these compounds. The BCCP, as a part of the ACCase moiety, isolated from a wide variety of organisms was shown to have great homology (16). It is important to investigate the

changes that occurred in this relatively conserved enzyme in the Poaceae species during evolution resulting in such sensitivity to cyclohexen-ones and PCAs. The information concerning the molecular differences of ACCase between species and the homology of ACCase from different species of organisms may help to shed light on the evolutionary relationships among these species. Information on the mechanism(s) of molecular binding of ACCase with cyclohexen-ones and PCAs could be very valuable for future genetic engineering of Poaceae crops such as corn, sorghum, and cereals facilitating the transfer of herbicide tolerance into these crops. If tolerance to the cyclohexen-one and PCA herbicides can be successfully transferred into important crops of the Poaceae, the use of these compounds could then be greatly expanded and herbicidal selectivity further refined.

Clofop-isobutyl (HCG 004) (12) is structurally analogous to diclofop-methyl and is active as a hypolipidemic drug in animals. The high potency of diclofop-methyl on grasses and low mammalian toxicity is a favorable character of this compound. There are a number of monoaromatic derivatives, such as salicylic acid, benfluorex, benzoate, *meta*-hydroxybenzoate, *para*-hydroxybenzoate, *para*-butylbenzoate, *para*-aminosalicylate, clofibrate, halofenate, and  $\alpha$ -cyano-4-hydroxycinnamate reported to inhibit fatty acid biosynthesis in animal systems (1). The inhibitory action on acetyl CoA carboxylase exerted by these compounds was proposed, although the molecular basis for the sensitivity of ACCase in animal systems is not clear. These compounds and their derivatives may have a similar inhibitory effect on ACCase in plants, analogous to clofop-isobutyl and diclofop-methyl. The potential use of these chemicals as herbicides should be evaluated.



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## 8.0 SUMMARY AND CONCLUSION

The physiological and morphological effects, mechanism of action, and selectivity of cycloxydim were investigated with the emphasis on root systems. Root materials ranging from intact roots of seedlings to root extracts were examined. Sethoxydim was used as a treatment control in some experiments, as the mode of action of this structurally similar compound has been demonstrated at the enzyme level.

Root materials of corn were sensitive to cycloxydim within micromolar concentration ranges, whereas soybean roots appeared to be tolerant to cycloxydim at relatively high (10 to 100  $\mu\text{M}$ ) concentrations. Adverse effects of cycloxydim at 1  $\mu\text{M}$  concentration on corn root materials but not soybean root materials are listed below sequentially based on the length of time required for the occurrence.

1. Inhibition of ACCase from root extracts of corn shown in a 15-minute assay.
2. Inhibition of  $^{14}\text{C}$  incorporation into lipid in excised root tips during 4 hours incubation in  $^{14}\text{C}$ -glucose and -acetate.
3. Synthesis of phosphatidylethanolamine (a fatty acid containing lipid) was inhibited while synthesis of sterols was not reduced in treated roots 4 hours after incubation with  $^{14}\text{C}$ -acetate.
4. Strong inhibition of root growth within 12 hours in tissue culture.
5. Inhibition of root growth of a whole seedling 1 day after treatment.

6. Cell vacuolization observed in treated roots 1 day after treatment.
7. Reddening of treated tissues occurred in meristematic tissues following the complete inhibition of root growth a few days after the treatment.

With an increased concentration of cycloxydim, the root growth of corn seedlings was inhibited after a shorter time of incubation and more severe cell and tissue injury in the root tip was observed than when roots were treated with cycloxydim at lower concentrations. Reddening was the last symptom observed in the whole course of cycloxydim-induced injury. Thus, tissue reddening evidently cannot be the direct effect of the herbicide, but it is rather a consequence of the inhibition of lipid synthesis and root growth which occurred earlier.

Reddening symptoms in roots developed as a band located at 1-2 mm from the root tip. Some researchers attempted to relate this reddening phenomenon to an increase in sugar content and the subsequent formation of anthocyanin. Data from the experiment reported here with  $^{14}\text{C}$ -UL-glucose did not directly confirm that hypothesis. In order to demonstrate this effect a much longer incubation would be required, which may in turn make interpretation even more complex due to the involvement of both primary and secondary metabolism.

Water-soluble carbohydrates are transported and accumulated in meristematic tissues. Data demonstrated that glucose is metabolized slowly and mono- and disaccharides were the predominant carbohydrates present in root tissues in tissue culture. The inhibitory effect of cycloxydim on lipid synthesis that leads to cell and tissue injury may subsequently induce secondary metabolic events involved, e.g., in healing, defense, and senescence. The development of reddish coloration may be an indirect result of these sequential but combined effects of cycloxydim.

Meristematic tissues are responsible for root elongation and growth. The inhibition of root growth and the specific location of the development of reddening symptoms in this tissue indicate that root meristems are an important site of action of cycloxydim. The accumulation of cycloxydim in root meristems, strong metabolic sinks, and the abundant presence of the herbicide target, ACCase, in this tissue may account for the apparent sensitivity of this tissue to cycloxydim. The

observed lack of sensitivity of soybean root ACCase to cycloxydim also strongly confirmed the herbicide selectivity between grasses (i.e. corn) and broadleaf species (i.e. soybean).

The root system as demonstrated in this study appears to represent an effective system for rapid bioassays, and physiological, morphological, and mode of action studies of herbicides that exhibit activity as lipid inhibitors. Results reported here showed corn roots, achlorophyllous plant material, expressing high sensitivity to cycloxydim. The inhibition of photosynthesis reportedly caused by other compounds with similar herbicidal activity to that of cycloxydim are probably secondary effects.

However, when using this root system for studies into the biochemical effects of cycloxydim on  $^{14}\text{C}$ -UL-glucose and -acetate, difficulty was encountered in the quantification of water-soluble compounds because of high variability. Factors potentially causing such variation include the gradient effects of cycloxydim resulting in varying injury patterns in the root tissues (based on the anatomical evidence), and losing radioactive compounds through the cut surface and through cells that were severely injured during rinsing. Glucose in root tips is not actively and quickly used and metabolized into compounds involved in glycolysis, TCA cycle, and lipid synthesis. In addition, it becomes economically infeasible and technically difficult to collect a large amount of meristematic tissue needed for an extensive TLC analysis. These potential drawbacks may limit the use of this technique for extensive study of glucose metabolism affected by chemical compounds in the future.

Consistent with results reported by other workers on the mode of action of cyclohexanediones and PCAs with the use of different plant parts, cycloxydim, similar to sethoxydim, functions primarily as an ACCase inhibitor in these studies using root tissue. The part of chemical structures in which sethoxydim and cycloxydim are different evidently is not involved in nor responsible for herbicidal activity. Root systems of etiolated seedlings could be used effectively for rapid evaluation of activity of postemergence grass herbicides and for a quick examination of the tolerance or susceptibility of species of particular interest.

## Vita

Hwei-Yiing Li was born on June 28, 1956, in Taipei, Taiwan, Republic of China. She received her B.S. from National Taiwan University, Taipei, Taiwan, R.O.C. in June 1979, majoring in Agronomy with an emphasis on crop physiology. In August 1979, she went to the Ohio State University, Columbus, Ohio, for graduate study in the Department of Agronomy. She completed her M.S. program in June, 1981. The title of her thesis was "The Autotoxicity of Alfalfa (*Medicago sativa*) on Seed Germination and Seedling Development". During the stay at OSU she assisted Dr. V. Raghavan, Department of Botany, in anther culture and thin-sectioning microtechniques. She then moved to Fayetteville, Arkansas, to begin her doctoral work at the University of Arkansas. She completed most of her course work between August, 1982 and May, 1985. She moved to Virginia and continued her doctoral work under Dr. C. L. Foy from August 1986 to July 1990 in the department of Plant Pathology, Physiology, and Weed Science at Virginia Polytechnic Institute and State University, Blacksburg, Virginia. She also worked part time for Dr. R. White in the Department of Biochemistry and Nutrition as a laboratory technician from July 1987 to December 1989, participating in studies of coenzyme isolation, purification, and biosynthesis in methanogen bacteria.

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