

# Effects of the Herbicide Sethoxydim and the Safener Dichlormid on Lipid Synthesis and Acetyl-CoA Carboxylase Activity of Grain Sorghum\*

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Sethoxydim, Dichlormid, Grain Sorghum, *Sorghum bicolor* (L.) Moench, Acetyl-CoA Carboxylase

The effects of individual or combined treatment of the cyclohexanedione herbicide sethoxydim and the safener dichlormid on total lipid synthesis, protein synthesis and acetyl-CoA carboxylase (ACCase, EC 6.4.1.12) activity of grain sorghum [*Sorghum bicolor* (L.) Moench, var. G 623] were investigated. Sethoxydim and dichlormid were tested at concentrations of 0, 5, 50, and 100  $\mu\text{M}$  each. Sethoxydim applied alone at 50 and 100  $\mu\text{M}$ , inhibited the incorporation of [ $^{14}\text{C}$ ]acetate into total lipids of sorghum leaf protoplasts by more than 50%, following a 4 h incubation. Dichlormid antagonized partially the inhibitory effects of sethoxydim on the incorporation of acetate into total lipids of sorghum protoplasts only when it was used at 100  $\mu\text{M}$ . Sethoxydim applied alone inhibited the incorporation of [ $^{14}\text{C}$ ]leucine into sorghum leaf protoplasts only at 100  $\mu\text{M}$ . Dichlormid was not inhibitory of this process at any concentration. The combined effects of sethoxydim and dichlormid on this process were mainly additive indicating no interactions of the two chemicals. Sethoxydim applied alone at 5 and 50  $\mu\text{M}$  inhibited the activity of ACCase extracted from leaf tissues of grain sorghum seedlings by 58 and 90%, respectively. Addition of the safener dichlormid to the assay medium did not inhibit ACCase activity of sorghum leaves even at the high concentration of 50  $\mu\text{M}$ . The combined effects of sethoxydim and dichlormid on the activity of sorghum ACCase were similar to those observed when sethoxydim was used alone. These results indicate that the protection conferred by dichlormid on grain sorghum against sethoxydim injury can not be explained on the basis of an antagonistic interaction of the two chemicals on target metabolic processes (lipid synthesis) or target enzymes (ACCase).

## Introduction

Sethoxydim is a member of the cyclohexanedione class of herbicides which are used for the selective control of grass weeds in dicotyledonous crops [1]. Grass crops such as grain sorghum [*Sorghum bicolor* (L.) Moench] and maize (*Zea*

*mays* L.) are also susceptible to preemergence or postemergence applications of this herbicide [1].

Recent advances in our understanding of the mechanism of action of cyclohexanedione herbicides have demonstrated that the molecular target site affected by these herbicides is the enzyme acetyl-CoA carboxylase (ACCase, EC 6.1.4.2) [2–4]. ACCase catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA [5]. This herbicide-mediated depletion of malonyl-CoA is thought to account for the inhibition of acetate incorporation into free fatty acids observed in chloroplasts or root tips of grass plants treated with sethoxydim or other cyclohexanedione herbicides [6, 7]. Aryloxyphenoxypropionate herbicides, although structurally unrelated to cyclohexanediones, comprise the second important class of postemergence applied gramini- cides which act as inhibitors of ACCase [6, 8]. The differential sensitivity of ACCase activity from tolerant dicots and susceptible monocots to cyclohexanediones and aryloxyphenoxypropionates has been established as the basis for the observed selec-

**Abbreviations:** ACCase, acetyl-coenzyme A carboxylase (EC 6.4.1.12); dichlormid, 2,2-dichloro-N,N-di-2-propenylacetamide; fluxofenim, O-[1,3-dioxolan-2-yl-methyl]-2,2,2-trifluoro-4'-chloroacetophenone-oxime; LSC, liquid scintillation counting; oxabetrinil,  $\alpha$ -(1,3-dioxolan-2-yl-methoxy)-iminobenzeneacetonitrile; PPF, Photosynthetic photon flux density; R-28725, 3-(dichloroacetyl)-2,2-dimethyl-1,3-oxazolidine; sethoxydim, 2-[1-(ethoxymino)butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one.

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tivity of these herbicides [2–8]. Alterations of ACCase that confer tolerance to grass species including maize have been reported recently by Parker *et al.* [9].

Dichloroacetamide safeners such as dichlormid and R-28725 have been reported to offer partial to good protection on maize and grain sorghum against injury caused by preemergence applications of low rates of the graminicide sethoxydim [10, 11]. The physiological basis of these antagonistic interactions between sethoxydim and dichlormid has not been established.

Therefore, the objectives of the research reported in this paper were to determine the individual or combined effects of sethoxydim and dichlormid on a) the incorporation of radiolabeled acetate and leucine into total lipids and proteins of leaf mesophyll protoplasts of grain sorghum and b) ACCase activity of grain sorghum seedlings.

## Materials and Methods

### Chemicals

Analytical grade (95% pure) samples of sethoxydim and dichlormid were obtained from BASF Wyandotte Corp., Parsippany, New Jersey, and Stauffer Chemical Co., Richmond, California, respectively. Radiolabeled samples of acetate, leucine, and sodium bicarbonate were purchased from ICN Biomedicals, Inc., Costa Mesa, California. All other chemicals and reagents used were obtained from various commercial sources.

### Plant material

Seeds of grain sorghum [*Sorghum bicolor* (L.) Moench, var. G623] were grown in plastic cups (473 ml) filled with a mixture of potting medium (Weblite Corp., Blue Ridge, Virginia), vermiculite, and sphagnum peat moss in a 2:2:1 (v/v/v) ratio. Limestone and a controlled release fertilizer were added to the soil mixture to supplement nutrient levels. Emerged seedlings were watered daily with tap water and grown in a growth chamber with a photosynthetic photon flux density (PPFD) of 300  $\mu\text{mol}/\text{m}^2/\text{s}$ , 16 h of light at 30 °C, and 8 h of dark at 20 °C. Leaves from 10 to 20 days old sorghum seedlings were then used for protoplast isolation or for the extraction of ACCase.

### Protoplast isolation

Leaf mesophyll protoplasts of sorghum were isolated enzymatically following the procedures of Zama and Hatzios [12]. Leaf segments of about 0.5 mm were prepared with a sharp razor blade and 4–5 g of cut leaves were infiltrated with a digestion medium containing 2% (w/v) cellulase (Cellulysin, Calbiochem, La Jolla, California), 0.5% pectinase (macerase, Calbiochem), 0.5 M sorbitol, 1 mM  $\text{CaCl}_2$ , 0.5% (w/v) BSA, and 5 mM MES-KOH buffer, adjusted to pH 5.5. The infiltrated tissue was then digested for 4 h under low light conditions and with a slow agitation in 60 ml of fresh digestion medium. The digested tissue was then filtered through an 80  $\mu\text{m}$  nylon net to remove the bundle sheath strands and debris. The filtrate was purified through a series of centrifugation steps after suspension in specific purification media composed of sorbitol and/or sucrose,  $\text{CaCl}_2$ , and MES-KOH buffer (pH 6.0). Details on these procedures have been published previously [12]. The protoplast preparation was then diluted to the desired volume with ice-cold dilution medium consisting of 0.4 M sorbitol, 1 mM  $\text{MgCl}_2$ , 1 mM EDTA, 2 mM  $\text{KH}_2\text{PO}_4$ , and 50 mM MES-KOH (pH 6.0). The chlorophyll content of isolated protoplasts was determined by the method of Arnon [13]. The average chlorophyll content of sorghum leaf mesophyll protoplasts used in this study was 15  $\mu\text{g}/\text{ml}$  of assay medium. The morphological integrity and purity of the isolated sorghum protoplasts were determined by light microscopic examination.

### Interactions of sethoxydim and dichlormid on the incorporation of radiolabeled precursors into sorghum leaf protoplasts

These experiments were conducted following the procedures described by Ashton *et al.* [14] and Zama and Hatzios [12]. Sethoxydim and dichlormid were tested at final concentrations of 0, 5, 50, and 100  $\mu\text{M}$  each. Assays were conducted in 25 ml Erlenmeyer flasks containing 2 ml of protoplast suspension (30  $\mu\text{g}$  chlorophyll), 0.05 ml of sethoxydim, 0.05 ml of dichlormid, and 0.1 ml of the appropriate radiolabeled precursor containing 1  $\mu\text{Ci}$  of radioactivity. The radiolabeled precursors used included L-[U- $^{14}\text{C}$ ]leucine (spec. act. 276 mCi/mol) for protein synthesis and [1,2- $^{14}\text{C}$ ]sodium ace-

tate (spec. act. 56.2 mCi/mmol) for lipid synthesis. Flasks with assay mixtures were sealed and incubated at 30 °C for 4 h in a shaking water bath (30 strokes/min), illuminated from above with a combination of incandescent and fluorescent lamps supplying 60  $\mu\text{mol}/\text{m}^2/\text{s}$  of PPFD at the levels of the flasks. At the end of incubation period, samples were collected and processed as previously described [12, 14]. Analysis by liquid scintillation spectrometry was then used to determine the incorporation of leucine into proteins and of acetate into total lipids of sorghum protoplasts. Data presented are the means of two experiments with two replications per experiment.

#### *ACCase extraction and assay*

ACCase was extracted from leaves of 10 days old sorghum seedlings grown in a growth chamber under the conditions described earlier in this section. Harvested leaves were frozen in liquid nitrogen and pulverized using a mortar and pestle. The exact procedures for the extraction and purification of sorghum ACCase have been described in detail by Secor and Cseke [15] and by Yenne and Hatzios [16]. Protein content was determined by using the Coomassie blue G-250 assay method described by Bradford [17].

ACCase was assayed in microcentrifuge tubes under a fume hood following the procedures of Secor and Cseke [15]. The final reaction volume was 250  $\mu\text{l}$ . The reaction mixture contained 50 mM tricine-KOH (pH 8.3), 5 mM  $\text{MgCl}_2$ , 2 mM dithiothreitol, 2 mM ATP, 15 mM  $\text{NaH}^{14}\text{CO}_3$  (0.33  $\mu\text{Ci}/\mu\text{mol}$ ), 1.5 mg protein/ml, and the inhibitor. Sethoxydim and dichlormid were used at 5 and 50  $\mu\text{M}$ , alone or in combination with each other. The reaction mixture was centrifuged for 30 sec and then preincubated for 15 min at 35 °C in a water bath. After this preincubation, 0.3 mM of acetyl-CoA was added to the assay mixture to initiate the reaction and 15 min later, the reaction was terminated by the addition of 50  $\mu\text{l}$  of 6 N HCl. A 150  $\mu\text{l}$  subsample from the reaction mixtures was placed into 4 ml scintillation vials which were then placed in a heating block (90 °C) for 1 h to dry the sample. Any unreacted  $^{14}\text{CO}_2$  was volatilized by the heat and adsorbed in an ascarite trap. The dried sample was suspended with distilled water (250  $\mu\text{l}$ ) and scintillation cocktail (3 ml) for radioactivity meas-

urements by LSC. The experiment contained three replicates and was repeated in time.

## **Results and Discussion**

The results of the experiments conducted in this study are presented in Fig. 1 and 2 and in Table I.

Data in Fig. 1 show that sethoxydim inhibited the incorporation of radiolabeled acetate into total lipids of sorghum leaf protoplasts in a concentration-dependent fashion. These effects were particularly evident with the two highest concentrations of sethoxydim tested (50 and 100  $\mu\text{M}$ ) which inhibited this process by about 50% (Fig. 1). A slight, but statistically significant inhibition of this process by the high concentrations of the safener dichlormid was also evident (Fig. 1). The effects of the combined treatments of 5 and 50  $\mu\text{M}$  of dichlormid with all concentrations of sethoxydim of the incorporation of acetate into total lipids of sorghum protoplasts appeared to be additive (Fig. 1). However, dichlormid at 100  $\mu\text{M}$  antagonized partially the effects of the high concentrations of sethoxydim on this process.

Data in Fig. 2 show that sethoxydim applied alone did not influence significantly the incorporation of radiolabeled leucine into TCA-precipitated proteins of sorghum leaf mesophyll protoplasts. A significant inhibition of this process was observed only when sethoxydim was used at 100  $\mu\text{M}$ . Treatments with 50 and 100  $\mu\text{M}$  of the safener dichlor-

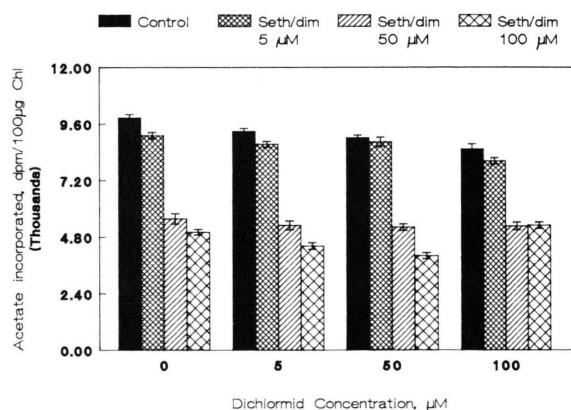


Fig. 1. Influence of individual or combined treatments with sethoxydim and dichlormid on the incorporation of [ $^{14}\text{C}$ ]acetate into total lipids of sorghum leaf protoplasts after 4 h of incubation. Bars on each histogram represent the standard error of each mean.

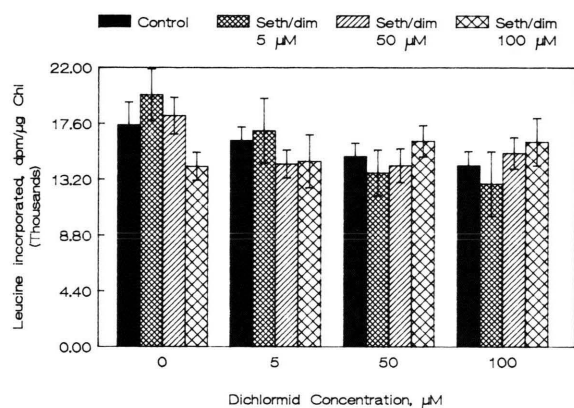


Fig. 2. Influence of individual or combined treatments with sethoxydim and dichlormid on the incorporation of [ $^{14}\text{C}$ ]leucine into sorghum leaf protoplasts after 4 h of incubation. Bars on each histogram represent the standard error of each mean.

mid caused also a slight inhibition of the incorporation of radiolabeled leucine into proteins of sorghum leaf protoplasts (Fig. 2). The combined effects of sethoxydim and dichlormid on this process were mainly additive. However, the combination treatment of 100  $\mu\text{M}$  of sethoxydim with 100  $\mu\text{M}$  of dichlormid appeared to be antagonistic.

Data in Table I show that sethoxydim applied alone at 5 and 50  $\mu\text{M}$  concentrations inhibited the activity of ACCase extracted from leaf tissues of grain sorghum seedlings by 58 and 90%, respectively. The  $I_{50}$  values of the inhibition of monocot ACCase activities by cyclohexanedione herbicides have been reported to range from 0.12 to 4.86  $\mu\text{M}$  [4]. Thus, the inhibitory effects of sethoxydim on the ACCase activity of sorghum leaves observed in

this study, are comparable to those reported in the literature.

Addition of the safener dichlormid to the assay medium did not inhibit ACCase activity of sorghum leaves even at the high concentration of 50  $\mu\text{M}$  (Table I). Thus, dichlormid seems to behave similarly to the oxime ether safeners, oxabetrinil and fluxofenim, which have been previously reported to be ineffective as modulators of sorghum ACCase activity [16].

The addition of sethoxydim/dichlormid combinations to the reaction mixture inhibited the activity of sorghum ACCase at levels similar to those obtained when the herbicide sethoxydim was used alone indicating that the combined effects of the herbicide and the safener on this enzyme are either additive or independent (Table I). Thus, it is obvious that the protection conferred by dichlormid on grain sorghum against sethoxydim injury can not be explained on the basis of an antagonistic interaction of the two chemicals on this herbicide-sensitive enzyme. These results appear to be similar to those reported for the herbicides bentazon and acifluorfen which are known to antagonize the efficacy of cyclohexanedione and aryloxyphenoxypropionate herbicides on grass weeds under field conditions [18]. Rendina and Felts [4] have reported that acifluorfen and bentazon were not inhibitors of ACCase activity in grasses and they did not appear to antagonize cyclohexanediones by interacting with them on this target enzyme.

## Conclusion

Overall, the results of the present study suggest that the dichlormid-mediated protection of grain

Table I. Effect of sethoxydim and dichlormid combinations on the *in vitro* activity of ACCase activity of sorghum leaves.

| Chemical       | Concentration [ $\mu\text{M}$ ] | ACCase activity [nmol $\text{CO}_2/\text{mg protein} \times \text{min}$ ] <sup>a</sup> | Percent inhibition [%] |
|----------------|---------------------------------|--|------------------------|
| Control        | 0                               | 100.8 $\pm$ 8.3  | 0                      |
| Sethoxydim (S) | 5                               | 42.3 $\pm$ 3.5   | 58                     |
| Sethoxydim     | 50                              | 9.8 $\pm$ 1.3  | 90                     |
| Dichlormid (D) | 5                               | 96.5 $\pm$ 7.8   | 4                      |
| Dichlormid     | 50                              | 89.4 $\pm$ 8.1   | 11                     |
| S + D          | 5 + 5                           | 45.8 $\pm$ 4.2   | 55                     |
| S + D          | 50 + 50                         | 11.5 $\pm$ 1.2   | 89                     |

<sup>a</sup> Mean values from three replications  $\pm$  SE of each mean.



sorghum against the herbicide sethoxydim cannot be explained on the basis of any antagonistic interactions between the safener and the herbicide at target metabolic processes. A safener-mediated alteration in the rate of uptake, translocation, and/or metabolism of sethoxydim by grass crops such as grain sorghum and maize may explain the protective effects of dichlormid in this situation and needs to be investigated in the future.

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