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Modes of Action of Selected Herbicides

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PREFACE

Western Regional Cooperative Research Projects on weeds (W-52, W-63, W-77) have been concerned with a wide range of herbicide-weed control problems over the years. These projects ranged from purely physiological investigations on translocation and mode of action of herbicides to practical matters such as environmental factors that alter herbicide efficiency. In the 60's, because of mounting public concern over immediate health hazards and long-term ecological consequences of pesticides in the environment, herbicidal research projects shifted in part away from studies of better crop protection toward assessment of the effect of herbicidal pollutants in the environment. Project W-108 was initiated to determine the effects of repetitive application or persistent pollution levels of herbicides on plant communities and individual species of the plant community. This bulletin deals with the effects or action of herbicides on the structure, chemical composition, and function of plant species.

Because many different herbicides were used in the project, the range of studies was correspondingly broad. Experiments were designed to study: (a) mechanism of action of several herbicides on dipeptidase synthesis in squash cotyledons; (b) effects of herbicide and growth regulators on bud sprouting of purple nutsedge; (c) cytological and biochemical effects of trifluralin on root tips; (d) inhibition of carotenoid synthesis by Sandoz 6706; and (e) mode of action of 2,4-D in stem tissue. Additional experiments dealt with the effects of growth regulators and other additives upon herbicide action.

The findings of this project may be used to develop principles that may enable man to avoid environmental pollution with herbicides while retaining their advantages. It is especially important to have an understanding of the effects of herbicides on plant biochemical processes so that this information may be related to the plant community and to ecological consequences. For example, a slight alteration of the photosynthetic efficiency of a plant species could have enormous consequences on a plant community and the whole ecosystem in which the plant community is found. Further, morphological and biochemical changes caused by herbicides may prove to be valuable indications of the presence of specific herbicidal pollutants in the environment.

P. G. Bartels and R. K. Nishimoto

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Chapter 1. Hormonal Control and Herbicidal Influence on Dipeptidase Synthesis in Squash Cotyledons

F. M. Ashton and R. Tsay

INTRODUCTION

Dipeptidase, one of the proteolytic enzymes of squash (*Cucurbita maxima* Duch. 'Hubbard') cotyledons, has been isolated and purified (Ashton and Dahmen, 1967). This enzyme has been shown to increase severalfold during seed germination. Its development was inhibited by protein synthesis inhibitors. *N*,6-benzyladenine (BA) stimulated development of the enzyme activity in half-seeds of squash (Sze and Ashton, 1971). However, these studies did not unequivocally show whether the dipeptidase was synthesized *de novo* or arose through activation of a zymogen. The inhibitor studies suggested that it was synthesized *de novo*. Specificity of control of the dipeptidase by plant hormones, effect of herbicides on its development, and the herbicidal action on hormonal control processes have not been studied.

The objectives of this study were to investigate (a) the effect of herbicides on dipeptidase activity, (b) the hormonal regulation of dipeptidase, (c) the influence of herbicides on hormonal control of dipeptidase, and (d) the *de novo* synthesis of dipeptidase. Some of the results reported in this paper have been previously published (Tsay and Ashton, 1971, 1974*a*, 1974*b*).

LITERATURE REVIEW

The effect of herbicides on various enzymes has been reviewed many times in the last two decades (Freed, 1953; Weintraub, 1953; Wort, 1954, 1964; Woodford et al., 1958; Hilton et al., 1963; Moreland, 1967; Robertson and Kirkwood, 1970; and Ashton and Crafts, 1973). Here the subject is limited to those papers concerning herbicides used in the study, namely: auxin, dichlobenil (2,6-dichlorobenzonitrile), endothall [7-oxabicyclo(2,2,1)heptane-2,3-dicarboxylic acid], and naptalam (*N*,1-napthylphthalamic acid). The term

"auxin," used as a generic term, includes synthetic and naturally occurring compounds having the growth-promotion properties of indol-3-acetic acid (IAA). The auxin compounds discussed herein are: 2,4-D [(2,4-dichlorophenoxy) acetic acid], dicamba (3,6-dichloro-*o*-anisic acid), and IAA. Because 2,4-D has also often been used at low concentrations as a hormone, it and other auxin compounds used as hormones are discussed as one topic, auxin. Dicamba used as a herbicide in most of the cases is discussed separately.

Auxin

The influences of auxin on different enzymes have been extensively studied since 1950, but these investigations have not shown a definite effect on the same enzyme from different plant materials. The different responses may relate to plant material itself, concentration of auxin used, time of treatment, and other factors.

Several investigators reported the effect of auxin on ribonuclease (RNase). Leo and Sacher (1970, 1971) reported that NAA (napthaleneacetic acid) at a rate of 10 $\mu g/\mu l$ inhibited increase of RNase in leaf sections of *Rhoeo discolor* and endocarp tissue sections of bean plants. In corn mesocotyl sections, however, 2,4-D increased the RNase activity at lower concentrations and inhibited enzyme activity at higher concentrations (Shannon et al., 1964). A ranking of species based on RNase is closely correlated with the ranking based on relative resistance to 2,4-D, according to Burkhalter and Carter (1969). Their results showed that changes in RNA and protein levels following 2,4-D treatments gave inconclusive results; however, RNase levels declined following treatment of sensitive species but rose in more resistant plants.

Many cell wall degradation enzymes have been found to increase in activity in response to auxin treatment. IAA treatment at the apices of decapitated-etiolated pea, bean, and corn seedlings increased the amount and specific activity of cellulase in adjacent tissue (Maclachlan et al., 1968). When applied to epicotyls of young peas, 10 ppm of auxin induced formation of cellulase, and this activity continued to increase with increasing auxin concentrations up to 5000 ppm (Fan and Maclachlan, 1966). However, in barley coleoptiles and pea epicotyl internode segments, cellulase was not induced by auxin; but beta 1,3- and/or beta 1,6-glucanase and hemicellulase activity increased after a 3-hour IAA treatment (Tanimoto and Masuda, 1968).

In slices of some plant tissue, invertase can be induced by washing. When slices of beet-root tissue were incubated in a solution of IAA or 2,4-D, invertase activity decreased (Palmer, 1967). However, in tissue slices of sugarcane, IAA and NAA initially enhanced invertase development, but later were inhibitory (Glasziou et al., 1966, 1968). Enzymes other than invertase in tissue slices were also reported to be influenced by auxins: NAA inhibited peroxidase synthesis in slices of sugarcane (Palmer, 1968), and a short-term exposure of auxin

to beet-root tissues and Jerusalem artichoke disks stimulated activities of acid phosphatase and ATPase (Glasziou et al., 1968).

The influences of auxin on other enzyme systems have also been studied. Auxin affected the activity of nitrate reductase in pea seedlings (Spesivtsev et al., 1970), glutathione reductase, and glucose-6-phosphate dehydrogenase in excised cotton cotyledons (Basler and Wills, 1968), ascorbase in bean (Panic and Franke, 1970), co-carbonylase in pea root tips (Kim and Bidwell, 1967), and aldolase, aminopeptidase, as well as glutamate: oxalacetate transminase in *Atropa belladonna* (Simla and Sopanen, 1971).

In an *in vivo* system, 2,4-D induced additional chromatin-bound RNA polymerase formation in soybean hypocotyls (O'Brien, 1967). At herbicidal concentrations, however, 2,4-D and IAA inhibited DNA synthesis by *Escherichia coli*, DNA polymerase supported by free DNA chromatin, or nucleohistone prepared from pea embryos (Schwimmer, 1968). Auxin was also found to be involved in the activation of enzyme activity. Citrate synthetase isolated from corn scutella, cauliflower, and bean plants could be activated by IAA *in vitro* and activation occurred at the external SH (sulfhydryl) group of the enzyme (Sarkissian, 1968, 1972).

Dicamba

Moreland et al. (1969) reported that dicamba inhibited GA-induced (gibberellic acid) alpha-amylase synthesis in distal-halves of barley seeds. In cotyledons of germinating squash seeds, dicamba at 10^{-3}M inhibited development of proteinase activity slightly (Ashton et al., 1968).

Dichlobenil

In barley seeds, dichlobenil was reported to inhibit development of GA-induced alpha-amylase (Penner, 1968; Devlin and Cunningham, 1970) and phytase (Penner, 1968); when applied to germinating squash seeds, it severely inhibited seedling growth and development of proteinase activity (Ashton et al., 1968). If BA is added to the dichlobenil treatment, some of the proteinase-activity inhibition could be nullified (Penner and Ashton, 1968).

Endothall

Endothall inhibited development of alpha-amylase in the endosperm of germinating barley seeds (Penner, 1968). It also depressed proteolytic enzymes in germinating squash seeds (Ashton et al., 1968), and this depression was not relieved by addition of BA (Penner and Ashton, 1968).

Naptalam

Naptalam had little effect on development of proteinase activity in germinating squash seeds (Ashton et al., 1968). Its influence on other enzyme systems has not been reported.

EFFECT OF HERBICIDES ON DIPEPTIDASE ACTIVITY

Protein is a major storage reserve in many plant seeds. This storage protein is hydrolyzed during seed germination by proteolytic enzymes, and the hydrolytic products are used by the developing seedling for synthesis of new structural and functional proteins and for energy. Inhibition of these enzymes by herbicides could have a profound effect on seedling growth and development. Proteinase activity in the cotyledon of squash seeds increases severalfold during seed germination (Wiley and Ashton, 1967). One or more proteinases probably are involved in this increase (Penner and Ashton, 1967), and some herbicides inhibit it (Ashton et al., 1968).

A dipeptidase—a proteolytic enzyme that hydrolyzes dipeptides but not proteins—has been isolated from cotyledons of squash seedlings (Ashton and Dahmen, 1967). Activity of this enzyme increased severalfold during germination (Sze and Ashton, 1971). This dipeptidase and the proteinase (Penner and Ashton, 1966, 1967) are probably synthesized *de novo*.

The purpose of this experiment was to survey the herbicides used in the proteinase study (Ashton et al., 1968) for their effect upon dipeptidase activity and to compare their effect on the development of these two enzymes.

Results and Discussion

Dipeptidase activity of squash cotyledons increases during germination (Sze and Ashton, 1971). However, when seeds were germinated in various herbicide solutions, the development of dipeptidase activity was altered by several of the herbicides (Table 1–1). Herbicides that inhibited development of dipeptidase activity by 70 percent or more were 2,4-D, dicamba, fenac [(2,3,6-trichlorophenyl)acetic acid], and naptalam. Those inhibiting by 63 to 34 percent were picloram (4-amino-3,5,6-trichloropicolinic acid), dichlobenil, bromoxynil (3,5-dibromo-4-hydroxybenzonitrile), amitrole (3-amino-*s*-triazole), atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine], endothall, and bromacil (5-bromo-3-*sec*-butyl-6-methyluracil). Those causing only slight inhibition, 24 to 16 percent, were diphenamid (*N,N*-dimethyl-2,2-diphenylacetamide), chlorpropham (isopropyl *m*-chlorocarbanilite), and trifluralin (α,α,α-trifluoro-2,6-dinitro-*N,N*-dipropyl-*p*-toluidine). Monuron [3-(*p*-chlorophenyl)-1,1-dimethylurea] was essentially the same as the control. However, dalapon (2,2-dichloropropionic acid), bensulide [*O,O*-diisopropyl phosphorodithioate *S*-ester with *N*-(2-mercaptoethyl)benzenesulfonamide], CDAA (*N,N*-diallyl-2-chloroacetamide), and CDEC (2-chloroallyl diethyldithiocarbamate) were somewhat stimulatory.

Herbicides that inhibited dipeptidase activity do not necessarily affect proteinase activity (Ashton et al., 1968) to the same degree. In general, herbicide

Table 1-1. Dipeptidase and proteinase activity in cotyledons of squash seedlings germinated in various herbicidal solutions

Herbicide treatment	Concentration	Dipeptidase activity, percent of control	Proteinase activity, percent of control[1]
2,4-D	10^{-3} M	6	45
Dicamba	10^{-3} M	17	86
Fenac	10^{-3} M	24	78
Naptalam	10^{-3} M	30	93
Picloram	10^{-3} M	37	79
Dichlobenil	10^{-4} M	39	30
Bromoxynil	10^{-4} M	44	32(78)
Amitrole	10^{-3} M	49	83
Atrazine	10^{-4} M	61	91
Endothall	10^{-3} M	64	70
Bromacil	10^{-3} M	66	99
Diphenamid	10^{-4} M	76	97
Chlorpropham	5×10^{-4} M	79	18(80)
Trifluralin	1.5×10^{-6} M	84	59
Monuron	10^{-4} M	105	93
Bensulide	5×10^{-5} M	112	35(92)
CDAA	10^{-3} M	119	58
CDEC	10^{-4} M	120	23(86)
Dalapon	10^{-3} M	124	98
Control	....	100	100

[1]From Ashton et al. (1968). Figures in parentheses are from Ashton and DeWitt (1971).

inhibition of development of dipeptidase activity was greater than inhibition of proteinase activity. This was true with 10 of the 19 herbicides evaluated: amitrole, naptalam, atrazine, dicamba, bromoxynil, bromacil, diphenamid, 2,4-D, fenac, and picloram. A notable exception was CDAA, which inhibited proteinase activity but did not inhibit dipeptidase activity. The degree of inhibition of the development of these two types of activity was similar for several herbicides: bensulide, chlorpropham, dichlobenil, endothall, and monuron. The data of Ashton and DeWitt (1971) for proteinase activity were used for the above comparisons for bromoxynil, bensulide, CDEC, and chlorpropham because they used water only as a solvent for these herbicides, whereas a small percentage of ethanol in water was used as a solvent for these four herbicides in the Ashton et al. (1968) study. Alcohol increased the inhibitory effect of these four herbicides on proteinase activity, even though alcohol at the concentrations used had no effect on the activity in the absence of herbicides. The influence of alcohol is considered to be related to increased membrane permeability, which resulted in higher concentrations of these herbicides in the cell.

The proteinase isolated by Penner and Ashton (1967) and the dipeptidase characterized by Ashton and Dahmen (1967) are probably the principal enzymes responsible for proteinase and dipeptidase activity measured in these studies. The activity of both of these enzymes increases with time during germination. They appear to be synthesized *de novo* and their synthesis controlled by a stimulus, probably a cytokinin, from axis (Penner and Ashton, 1966, 1967; Wiley and Ashton, 1967; Sze and Ashton, 1971). Therefore, inhibition of development of dipeptidase activity by the several herbicides could involve one or more mechanisms. Possibly, the herbicides interfere with development or transport of the hormone that controls synthesis of the dipeptidase in the cotyledons. Alternatively, they may interfere with synthesis of the dipeptidase in the cotyledons. Interference with dipeptidase synthesis could be brought about by inhibition of any of the several essential components of protein synthesis—e.g., DNA, RNA, ATP, or ribosomes.

Protein synthesis. Inhibition of protein synthesis in hemp sesbania (*Sesbania exaltata* (Raf.) Cory) hypocotyls and/or barley coleoptiles by chlorpropham, CDAA, and endothall were reported by Mann et al. (1965). Moreland et al. (1969) observed that protein synthesis in soybean hypocotyls was inhibited by chlorpropham, fenac, CDEC, dicamba, and dichlobenil. The results of these two studies are not in agreement with regard to the inhibition of protein synthesis by CDAA, CDEC, and dichlobenil. In the studies by Mann et al. (1965), CDEC and dichlobenil did not inhibit protein synthesis, whereas they did in the research reported by Moreland et al. (1969). Conversely, CDAA did not inhibit protein synthesis in the studies by Mann et al. (1965). We also find variation between proteinase and dipeptidase activity development in squash cotyledons with CDAA: it inhibits proteinase activity but not dipeptidase activity. Moreland et al. (1969) reported that some of the differences between their research and that of Mann et al. (1965) can possibly be attributed to differences in concentrations of the herbicides tested. However, differences in the solvent used to dissolve the herbicides, as well as species differences, also may have been involved. Mann et al. (1965) used ethanol and Moreland et al. (1969) used dimethyl sulfoxide (DMSO). Previously we discussed the influence of ethanol on the effect of bromoxynil, bensulide, CDEC, and chlorpropham on proteinase activity of squash cotyledons. The report by Mann et al. (1965) supports the concept that inhibition of development of dipeptidase activity in squash cotyledons by fenac, dicamba, dichlobenil, and endothall may be due to inhibition of protein synthesis. These investigators did not investigate the effect of 2,4-D, naptalam, bromoxynil, or bromacil on protein synthesis. Possibly, inhibition of dipeptidase activity by these herbicides is also via protein synthesis. However, one or both of these studies included picloram, amitrole, or atrazine, which did not inhibit protein synthesis; therefore, these herbicides must inhibit dipeptidase via some mechanism other than protein synthesis.

RNA synthesis. RNA synthesis probably is required for development of dipeptidase activity in squash cotyledons. Moreland et al. (1969) investigated the influence of several of the herbicides used in our study on RNA synthesis in corn mesocotyl sections (using ^{14}C-ortic acid incorporation) and in soybean hypocotyl sections (using ^{14}C-ATP incorporation). Only two of the herbicides that inhibited dipeptidase activity, and which were tested for RNA synthesis, inhibited RNA synthesis. Therefore, amitrole, dichlobenil, fenac, dicamba, and picloram inhibition of dipeptidase is probably not via the inhibition of RNA synthesis. Atrazine inhibited RNA synthesis in the ^{14}C-ATP assay, but was stimulatory in the ^{14}C-orotate assay and inhibited dipeptidase activity. Therefore, the inhibition of dipeptidase activity by atrazine may be via RNA synthesis. Chlorpropham inhibited RNA synthesis very significantly in both assays, but had only a slight inhibitory effect on dipeptidase activity. Other herbicides that inhibited dipeptidase activity, but that were not tested for their influence on RNA synthesis and therefore could be acting via RNA, are 2,4-D, naptalam, bromacil, endothall, and bromoxynil.

ATP synthesis. Compounds that interfere with ATP production by acting as uncouplers of oxidative phosphorylation or as electron transport inhibitors could inhibit development of dipeptidase activity. Herbicides that inhibit dipeptidase activity, and which have been reported to uncouple oxidative phosphorylation, are 2,4-D (Switzer, 1957; Lotlikar et al., 1968), naptalam (Lotlikar et al., 1968), dichlobenil (Foy and Penner, 1965), and dicamba (Foy and Penner, 1965).

In a study on the influence of dichlobenil, endothall, and bromoxynil on cytokinin control of proteolytic activity in squash cotyledons, it was suggested that dichlobenil inhibited cytokinin synthesis or transport, endothall inhibited RNA metabolism, and bromoxynil interfered with the cytokinin system and protein synthesis (Penner and Ashton, 1968).

Conclusions

Dipeptidase activity of cotyledons of squash increased during germination. Several herbicides inhibited this increase to various degrees. Herbicides inhibiting development of dipeptidase activity by 70 percent or more were 2,4-D, dicamba, fenac, and naptalam. Those inhibiting dipeptidase activity by 63 to 34 percent were picloram, dichlobenil, bromoxynil, amitrole, atrazine, endothall, and bromacil. Those causing only slight inhibition, 24 to 16 percent, were diphenamid, chlorpropham, and trifluralin. Monuron was essentially the same as the control. However, dalapon, bensulide, CDAA, and CDEC were somewhat stimulatory. When compared with previously reported herbicide activity on proteinase activity, herbicides that inhibited dipeptidase activity did not necessarily affect proteinase activity to the same degree.

HORMONAL REGULATION OF DIPEPTIDASE

Hormonal regulation of dipeptidase in squash cotyledons was observed by Sze and Ashton (1971). They showed that squash seeds required the presence of the embryo axis for maximum development of dipeptidase activity, and that the cytokinin BA could replace the embryo axis. Proteinase isolated from the same tissue was found to be specifically controlled by cytokinin (Penner and Ashton, 1967). These two enzymes are closely related in protein digestion during seed germination. However, the properties and time-course studies of dipeptidase are different from those of proteinase. Dipeptidase may or may not show the same specificity. The question of whether dipeptidase is controlled by BA alone or is also controlled by other growth regulators was the subject of this investigation.

Results

Morphological response to hormones. All four hormones—BA, GA, IAA, and ABA (abscisic acid)—affected the growth of seedlings. No quantitative measurement of these responses was taken, but the responses are described below. These responses appeared to be more pronounced at the higher concentration (5×10^{-5} M) than at the lower concentration (5×10^{-6} M).

Intact seeds. BA-treated seeds had shortened hypocotyls, and the growth of the primary roots was greatly inhibited. IAA also reduced the growth of primary roots, as well as the development of root hairs. ABA almost stopped seed germination. However, GA promoted seedling growth; the seedlings had larger cotyledons, longer hypocotyls, and longer primary roots compared to the controls.

Distal-half cotyledons. BA, IAA, and GA induced the enlargement of distal-half cotyledons. Among these three hormones, BA showed the greatest effect. Distal-half cotyledons treated with BA were twisted and curled. IAA and GA increased the size without changing the form. Distal-half cotyledons incubated in ABA exhibited almost no expansion.

Effect of hormones on dipeptidase reaction. The enzyme was prepared in the usual manner by extraction from cotyledons of control plants grown in water. A quantity of hormone solution was added to the enzyme. Enzyme activity was then measured and compared to the control. The results showed that dipeptidase activity was not affected by the presence of hormones.

Effect of hormones on development of dipeptidase. Dipeptidase activity of squash cotyledons, either from intact seeds or excised cotyledons, increased during seed germination (Sze and Ashton, 1971). This increase can be altered by adding the hormones. The results are presented as a relative percentage of the control and were statistically analyzed by complete randomized design (Table 1–2). The control obtained from the intact seed without added hor-

Table 1-2. Effect of different hormones on development of dipeptidase activity during germination. The value obtained from intact seeds with water is 100 percent.

	Values expressed as relative percentages			
	BA	GA	IAA	ABA
Intact seeds[1]				
0 (control)	100.0a[2]	100.0d	100.0a	100.0a
5×10^{-6} M	88.6ab	93.5d	96.0ab	75.5b
5×10^{-5} M	66.3c	116.5c	72.5bc	42.5c
Distal-halves				
0	70.6bc	76.6c	74.5bc	71.5b
5×10^{-6} M	91.0[3]a	142.0b	50.5d	72.5b
5×10^{-5} M	106.0[3]a	175.0a	50.1d	49.0c

[1]After germination, distal-halves of intact seeds were harvested for enzyme preparation.
[2]Means followed by the same letters are not significantly different at the 5 percent level as determined by Duncan's Multiple Range Test. All comparisons are made within the same column.
[3]Enzyme activity was not corrected for volume increment; if this had been done, values would have been somewhat higher than those reported.

mones was arbitrarily set as 100 percent; the other values were calculated as a percentage of this value. The results of this experiment are presented in the following five sections.

Partial control of dipeptidase activity by embryo axis. The data from the experiments at zero concentration of hormones showed that enzyme activity of distal-half cotyledons was about 75 percent of that of the control (Table 1-2). Therefore, without the proximal half, which includes the embryo axis, enzyme activity was reduced about 25 percent. Since cotyledon tissues in both distal-half and proximal half are similar, the reduction of enzyme activity in distal-half suggests that part of the enzyme activity in the cotyledon is controlled by the embryo axis.

Effect of BA on dipeptidase: promotion and inhibition. The reduction of enzyme activity in distal-half cotyledons can be replaced by the addition of BA (Table 1-2). The results demonstrated that by supplying BA 5×10^{-5} M or 5×10^{-6} M the enzyme activity in distal-half cotyledons developed to the same level as in the control. However, at the higher concentration, 5×10^{-5} M, BA inhibited enzyme development in intact seeds. Similar observations were made by Sze and Ashton (1971).

Effect of GA on dipeptidase: promotion. GA showed a strong promotive effect on dipeptidase development in distal-half cotyledons (Table 1-2). By adding GA, enzyme activity in distal-halves increased drastically. As compared to the zero concentration, GA at both concentrations approximately doubled the enzyme activity. In intact seeds, GA also slightly enhanced enzyme activity at

the higher concentration. Thus GA, as well as BA, is a stimulus that can promote dipeptidase development and can replace the effect of the embryo axis.

Effect of IAA and ABA on dipeptidase: inhibition. In intact seeds, the lower concentration of IAA had no effect on the enzyme activity; at the higher concentration it was slightly inhibitory. For the distal-half cotyledons, both concentrations decreased dipeptidase development (Table 1–2). Results similar to IAA were also observed in auxin-type herbicides, dicamba, and 2,4-D; these are reported in Table 1–4. ABA slightly reduced the enzyme activity in intact seeds at the lower concentration, 5×10^{-6} M. At the higher concentration, 5×10^{-5} M, ABA decreased the enzyme activity about 60 percent. Enzyme activity in both intact seeds and distal-half cotyledons was about at the same level, except at zero concentration; there was no difference in activity between 5×10^{-6} M concentration and zero concentration in distal-half cotyledons. These results indicate that in the intact system the lower concentration of ABA stopped the influence of the embryo axis. The action of embryo axis was also stopped at the higher concentration of ABA and there was an additional inhibition of this enzyme's development with cotyledons *per se.*

Effect of BA + GA on dipeptidase. This experiment was designed to determine the interaction of GA and BA on the development of dipeptidase. Two concentrations of BA were combined with two concentrations of GA. The data from each combination were compared to the additive value of the individual components. If there was any additive or synergistic effect, the activity of a combination should be equal to or greater than the total activity of their components. Table 1–3 suggests that none of the combinations was additive or synergistic. Therefore, both BA and GA appear to act at the same biochemical site in this system.

Table 1–3. Effect of combination of BA and GA on development of dipeptidase activity, using distal-half cotyledons. Values are expressed as relative percentages.

Treatment	Value (%)
H_2O (control)	100.0e[1]
GA 5×10^{-6} M	224.6d
BA 5×10^{-6} M	232.0cd
GA 5×10^{-5} M	264.0bcd
BA 5×10^{-5} M	310.3ab
GA 5×10^{-6} M + BA 5×10^{-6} M	249.6bcd
GA 5×10^{-6} M + BA 5×10^{-5} M	298.0abc
GA 5×10^{-5} M + BA 5×10^{-6} M	241.0bcd
GA 5×10^{-5} M + BA 5×10^{-5} M	344.0a

[1]Means followed by the same letters are not significantly different at the 5 percent level as determined by Duncan's Multiple Range Test.

Discussion

Partial control of dipeptidase by the embryo axis. These results show that 25 percent of the dipeptidase activity of the intact seeds was controlled by the embryo axis for a 4-day germination period. For a 5-day germination, it controlled 40 percent of the activity (Sze and Ashton, 1971). Genetic differences may partly account for this apparent large difference in addition to the difference in age. Sze and Ashton suggested that a stimulus for the embryo axis was needed to initiate maximal enzyme activity, and the stimulus was found to be cytokinin. However, from the results of our experiment, it is concluded that GA as well as BA promoted the development of the activity of this enzyme. In addition, IAA, ABA, and other factors may be implicated in the regulation. Apparently, the function of the embryo axis in controlling this enzyme is quite complicated. In order to further understand this phenomenon, it would be necessary to know the interaction among the hormones, their quantitative changes during seedling development, and their distribution. Such studies were beyond the scope of our investigation.

Effect of hormones on development of dipeptidase. In distal-half cotyledons, development of dipeptidase was stimulated by GA and BA and inhibited by IAA and ABA. The experiment, which combined the two hormones BA and GA, was conducted to see if their combined action was additive or synergistic. Since their site of action (as outlined below) is different, one would assume that the action would be additive. The action of cytokinin on protein synthesis is thought to be associated with tRNA (Fox, 1966; Gefter and Russell, 1969). GA is believed to be involved in the production of mRNA molecules on DNA template (Chrispeels and Varner, 1967*a*). This information suggests that these two hormones do not act on the same site. Therefore, additive or synergistic effects appear possible when the two hormones are combined. However, in the current investigation, additive or synergistic responses were not found. This may be because there was considerable variation. The variation was not caused by changing the environment, because environmental conditions were kept constant, so it may be due to genetic differences. The phenotypic difference in texture and color of the seed coat was previously observed and described (Sze, 1970). Although seeds used were selected for uniformity, it was not possible to obtain identical experimental material for use in a given experiment. Squash, a monoecious plant, is often cross-pollinated, and hence certain variations in genotypes are produced. Therefore, endogenous factors involved in the synthesis of this enzyme can be altered and can cause differential development of dipeptidase activity. Other factors (e.g., herbicides and insecticides) also may have been involved before the seeds were brought into the laboratory.

Both GA and BA appear to induce development of dipeptidase activity in distal-half cotyledons, but they show different effects on the intact seeds. In intact seeds, GA promotes the enzyme activity and seedling growth slightly; BA inhibits these phenomena. These differences for these substances may be due to

their different physiological roles and finally are related to the endogenous hormonal balance. It has been suggested that cytokinin may counteract a germination inhibitor in barley seeds (Paleg, 1965). It has been shown that in the barley aleurone layer, the inhibition of alpha-amylase synthesis by ABA can be partially overcome by addition of a large amount of GA (Chrispeels and Varner, 1967*b*). In intact barley seeds, production of alpha-amylase is inhibited by ABA, and this inhibition is only slightly reversed by the addition of excess GA. However, when a cytokinin such as kinetin or BA is added to the inhibited system, the enzyme production, as well as germination, is almost fully recovered (Khan, 1969). Therefore, acting as an antagonist of a germination inhibitor, the cytokinins seem essential for the functioning of endogenous GA(s), the inducer(s) of alpha-amylase synthesis (Paleg, 1965). Khan (1969) reported that in embryo-less half-seeds kinetin failed to relieve ABA inhibition of alpha-amylase, but his data showed that by adding GA the alpha-amylase activity recovered to the same level as in the control. Thus, both BA and GA may counteract ABA inhibition. The interaction among these three hormones, GA, BA, and ABA, was not investigated in this study, but the antagonism may appear in this regulatory system.

IAA is incapable of causing an increase in the proteolytic activity (Penner and Ashton, 1967), but it inhibits development of dipeptidase in intact seeds and in distal-half cotyledons. The action of auxin has been reviewed by Key (1969); auxin causes an increase in DNA, RNA, and protein synthesis at hormonal levels. An inhibited development of dipeptidase activity by the higher concentration of IAA may not be due to auxin effects of DNA, RNA, or protein synthesis, but rather to the accumulation of inhibitory metabolites. Data presented in our report do not specifically suggest this, however.

Biochemical aspects of the action of ABA have been reviewed recently (Addicott, 1972). Many studies indicate that ABA noncompetitively interacts with other growth hormones. The results of this experiment are inadequate to account for a specific ABA-hormone interaction. However, ABA nullified the effect of the embryo axis at lower concentrations and stopped the further development of the enzyme activity in the cotyledon at the higher concentration. ABA may counteract the effect of BA or GA (as discussed above).

Conclusions

Dipeptidase activity of cotyledons of squash increased during seed germination. The embryo axis controlled about 25 percent of this development for 4-day germination. Four plant-growth regulators (GA, BA, IAA, and ABA), each with two concentrations, 5×10^{-6} M and 5×10^{-5} M, were examined for their effect on the dipeptidase activity. None of these regulators affected the activity *per se* of the isolated dipeptidase. The presence of the growth regulators during the germination process caused various degrees of effects on dipeptidase development. In intact seeds, BA and IAA inhibited dipeptidase activity at the

higher concentration, 5×10^{-5} M; ABA reduced the activity at both concentrations, 5×10^{-6} M and 5×10^{-5} M; GA, however, enhanced its development at the higher concentration, 5×10^{-5} M. In distal-half cotyledons, BA and GA both stimulated the enzyme development; however, in combination they showed no additive or synergistic effect. ABA suppressed the enzyme activity at the higher concentration, and IAA inhibited the enzyme activity at both levels.

INFLUENCE OF HERBICIDES ON HORMONAL CONTROL OF DIPEPTIDASE

Penner and Ashton (1967) reported that proteinase activity in squash cotyledons increased severalfold during germination, and that it was specifically controlled by cytokinin. Several herbicides inhibited the development of this proteinase activity (Ashton et al., 1968). BA could reduce the inhibition produced by dichlobenil and endothall (Penner and Ashton, 1968).

Dipeptidase, one of the proteolytic enzymes in squash cotyledons, also increases severalfold during the germination (Sze and Ashton, 1971). Results of previous experiments showed that dipeptidase might be controlled by GA and BA, and that the presence of different herbicides in the culture solution during germination caused various effects on development of this enzyme.

Five herbicides found to inhibit the development of dipeptidase in the cotyledons of intact seeds were: dicamba, 2,4-D, naptalam, dichlobenil, and endothall. The purpose of this experiment was to investigate whether these five herbicides might alter the hormonal control of dipeptidase development.

Results

Morphological response to herbicides. Both BA and GA stimulated the expansion of distal-half cotyledons. As compared to the control treatment, naptalam 10^{-4} M, 2,4-D 10^{-5} M, and dicamba 10^{-5} M had no effect on growth of this storage tissue, nor were toxic symptoms observed. When BA or GA was combined with one of the three herbicides, BA- or GA-stimulation was unchanged. However, dichlobenil completely stopped the growth of the distal-half cotyledons, and the effect of BA and GA on stimulation was also nullified. Additionally, dichlobenil caused exudation of a gummy material from the tissue.

Endothall injured the distal-half cotyledons—the margins and surfaces of this tissue seemed to be burned. Development was inhibited by endothall alone, and expansion induced by GA and BA was also reduced by endothall.

Effect of herbicides on enzyme reaction. Each herbicide was added to an enzyme solution prepared from control plants grown in water. Results showed that activity of the dipeptidase was not affected by the herbicides.

Table 1-4. Influence of herbicides on hormonal control of dipeptidase development. Values are expressed as relative percentages.[1]

Treatment	Value (%)
Individual treatment	
H_2O	100.0
GA 5×10^{-5} M	168.4
BA 5×10^{-5} M	155.3
Dicamba 10^{-5} M	58.5
2,4-D 10^{-5} M	69.3
Naptalam 10^{-4} M	67.0
Dichlobenil 10^{-4} M	87.5
Endothall 10^{-3} M	249.0
Combination treatment	
Dicamba 10^{-5} M + GA 5×10^{-5} M	73.2
Dicamba 10^{-5} M + BA 5×10^{-5} M	78.0
2,4-D 10^{-5} M + GA 5×10^{-5} M	98.3
2,4-D 10^{-5} M + BA 5×10^{-5} M	112.3
Naptalam 10^{-4} M + GA 5×10^{-5} M	134.0
Naptalam 10^{-4} M + BA 5×10^{-5} M	110.5
Dichlobenil 10^{-4} M + GA 5×10^{-5} M	55.0
Dichlobenil 10^{-4} M + BA 5×10^{-5} M	183.0
Endothall 10^{-3} M + GA 5×10^{-5} M	278.5
Endothall 10^{-3} M + BA 5×10^{-5} M	398.5

[1]Values of treatments of H_2O, GA 5×10^{-5} M, BA 5×10^{-5} M are the average of 20 replications. Values of remaining treatments are the average of four replications.

Effect of herbicides on hormonal control of dipeptidase development. Naptalam at 10^{-4} M and 2,4-D at 10^{-5} M reduced dipeptidase development activity significantly (Table 1-4). The addition of GA and BA completely nullified the inhibition produced by these two herbicides.

Dicamba at 10^{-5} M also significantly reduced enzyme development; however, only BA prevented this inhibition (Table 1-4). Effect of GA on inhibition was not significant.

Dichlobenil 10^{-4} M showed little inhibition of dipeptidase development (Table 1-4). The combination of BA and dichlobenil increased enzyme activity more than did the BA treatment; addition of GA to dichlobenil suppressed the enzyme development. Since the value of control treatments was much higher than usual in this experiment, the relative percentage of BA treatment was consequently lower. The effect of BA on the promotion of enzyme activity was masked by experimental error.

Endothall markedly effected dipeptidase development (Table 1-4), but a considerable variation in the data made it impossible to determine its interac-

tion with GA or BA. This also obscured the effect of BA on the promotion of dipeptidase. More replications are needed to reduce the experimental error.

Discussion

It is difficult to find any correlation between the enlargement of distal-half cotyledons and enzyme activity. This is probably because chemical treatments effected not only the synthesis of dipeptidase but also the many other metabolic reactions involved in the enlargement of this storage tissue. Interference of dipeptidase synthesis by these herbicides is probably due to their effect on one or more of at least three areas of metabolism: protein synthesis, RNA synthesis, or ATP synthesis. Time limitation for this research prevents investigation to determine the possible inhibition site(s) for each herbicide. However, different interactions altered the enzyme synthesis when plant-growth regulator BA or GA was added to the herbicides.

Dicamba and 2,4-D reduced the enzyme activity in distal-half cotyledons and intact seeds. The influence of these two auxin-type herbicides on dipeptidase development appears to be similar to IAA, a naturally occurring auxin. Addition of GA or BA to these two herbicides counteracts their inhibition to a certain degree (Table 1–4); the promotive effect of GA and BA on enzyme activity was also diminished by the herbicides. The interactions thus appear to be complicated and probably involve more than one of the components involved in the synthesis of this enzyme. Both 2,4-D and dicamba were found to inhibit the ATP synthesis (Lotlikar et al., 1968); however, in recent reviews (Key, 1969; Hanson and Slife, 1971; Ashton and Crafts, 1973), auxin has also been reported to cause an overproduction of RNA, to promote ethylene formation, and to change the pattern of development of endogenous cytokinin. In treated plants, 2,4-D was also found to change the amount of metallic ions, vitamins, and amino acids available for enzyme synthesis. While the major factor(s) in the interaction is not known, perhaps all of these factors are directly or indirectly involved in the action.

Naptalam also inhibited enzyme development in distal-half cotyledons and intact seeds. Addition of GA and BA to naptalam yielded results similar to those previously discussed for 2,4-D. Naptalam reportedly has little effect on protein synthesis in barley and sesbania seeds (Mann et al., 1965), but has been shown to inhibit oxidative phosphorylation in cabbage mitochondria (Lotlikar et al., 1968). Therefore, it is suggested that naptalam suppresses the dipeptidase mainly through the inhibition of ATP synthesis, and that it interacts with GA or BA at a different site.

Dichlobenil and endothall both inhibited the dipeptidase in intact seeds. However, dichlobenil had little effect on dipeptidase development in the distal-half cotyledons, and endothall increased enzyme activity in the tissue. This suggests that there is an interaction between the embryo axis and these two herbicides, although data obtained from the combination treatments were

so variable that no conclusion as to the interaction of two herbicides with the two hormones was possible. Apparently there was an unstable factor(s) interfering in these experiments. Further kinetics studies would be necessary to detect this factor.

Conclusions

Five herbicides were examined for their influence on dipeptidase activity and growth of the distal-half cotyledons of squash during incubation. None of these herbicides affected the enzymatic reaction *per se* of the isolated dipeptidase. Herbicides in the culture solution caused different effects on the growth and the development of dipeptidase activity, although it is difficult to find any correlation between growth of distal-halves and enzyme activity. Dicamba, 2,4-D, and naptalam showed no effect on growth but reduced enzyme development significantly. The addition of BA or GA relieved part of the inhibition produced by these three herbicides. Dichlobenil hardly inhibited dipeptidase activity but stopped growth of this tissue. Endothall damaged the distal-halves but increased enzyme activity. How these two herbicides interact with GA and BA is not known.

DE NOVO SYNTHESIS OF DIPEPTIDASE

Dipeptidase activity of squash cotyledons increased during seed germination and was strongly inhibited by cycloheximide, a protein synthesis inhibitor; *de novo* synthesis of this enzyme was suggested (Sze and Ashton, 1971). However, such an inhibitor study is in itself not proof of *de novo* synthesis of an induced enzyme. Activation of a latent enzyme may be indirectly dependent upon protein synthesis (Filner et al., 1969). The effect of protein synthesis inhibitors on activation of enzyme activity was shown by Shain and Mayer (1968) in an *in vivo* system. They found that protein synthesis inhibitors partially inhibited development of trypsin-like enzyme activity in lettuce seeds and amylopectin-1,6-glucosidase in pears.

The question of whether dipeptidase is synthesized *de novo* or activated during the seed germination was the purpose of the following experiment. The radioactive amino acid incorporation into protein method was used for this investigation.

Results

Seeds were germinated in water the first 96 hours and then placed in ^{14}C-leucine for 24 hours. Table 1–5 and Figure 1–1 show the results. Table 1–5 shows that the specific activity of the enzyme increased with each purification step, and Figure 1–1 shows that the peak of radioactivity was in excellent agreement with the peak of enzyme activity. The increase in the specific radio-

Table 1-5. Purification of dipeptidase and the incorporation of ^{14}C-leucine into the enzyme. Crude enzyme obtained from seeds incubated in ^{14}C-leucine for 24 hours after 4-day germination

Fraction	(1) Protein mg/ml	(2) Cpm/ml	(3) Specific radio-activity (2)/(1)	(4) Fold of specific radio-activity	(5)* Enzyme units per ml	(6) Specific enzyme activity (5)/(1)	(7) Fold of enzyme purifi-cation	(8) Percent of enzyme recovery	(9) Percent of radio-active recovery
Crude (I)	48.500	3,852	79.4	1.00	3.813	0.078	1.00	100.0	100.0
Acetone (II)	17.800	4,031	226.5	2.85	11.040	0.620	7.90	38.2	12.79
Sephadex (III)	1.831	666	363.7	4.57	3.120	1.704	21.70	50.4	8.96
DEAE (IV)	0.075	45	600.0	7.55	3.764	50.186	638.50	112.5	1.23

***1 unit** = 1 ml of 0.1 M KOH/60 minutes.

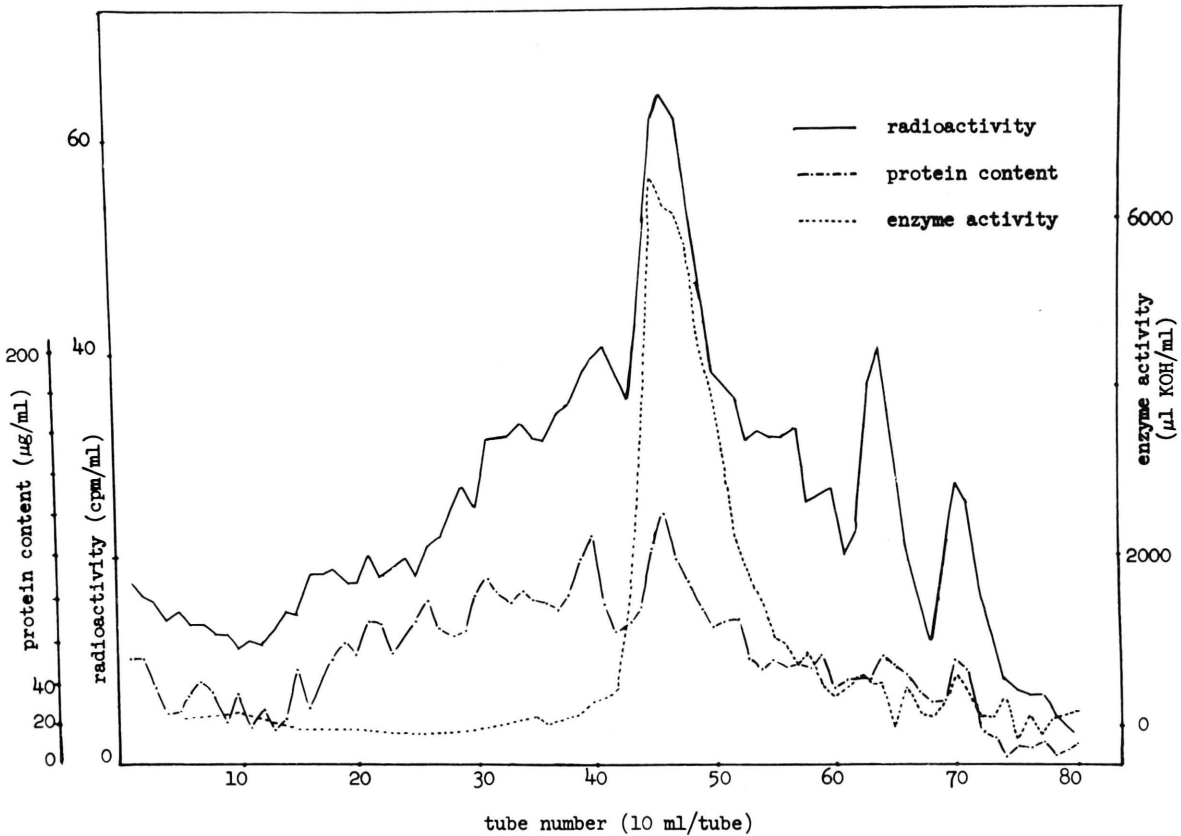

Figure 1–1. Amount of protein, enzyme activity, and radioactivity in the DEAE fraction. Enzyme source from seeds incubated in ^{14}C-leucine for 24 hours after 4-day germination.

activity during purification, as well as the peak of radioactivity in active enzyme region, provides strong evidence of *de novo* synthesis of this dipeptidase.

Discussion

In order to use the radioactive amino acid method to prove the *de novo* synthesis of an enzyme, it is necessary to show that (a) radioactive amino acid is incorporated into the enzyme protein, and (b) the amount of radioactive amino acid that is incorporated into the enzyme protein is greater than that found in other proteins. The data show that, in the DEAE fraction, ^{14}C-leucine is incorporated into the purified dipeptidase, and that there is a greater incorporation of ^{14}C-leucine into dipeptidase than into other proteins. Since ^{14}C-leucine can be metabolized during the incubation, the greater amount of ^{14}C-incorporation into the enzyme becomes the most important criterion for demonstration of the *de novo* synthesis of an enzyme. In this study, the greater amount of ^{14}C-incorporation is evident from the pattern of radioactive distribution in the DEAE fraction and from the comparison of specific radioactivity in each step of enzyme purification. This evidence is discussed in the following two paragraphs.

Radioactive distribution in DEAE fraction. Varner (1964) incubated embryo-less barley half-seeds in ^{14}C-phenylalanine solution for 18–20 hours, and alpha-amylase was then extracted and fractionated by DEAE chromatography. The result showed that, in the presence of GA, alpha-amylase constituted a major fraction of the radioactivity, and that, without GA, there was no radioactivity in alpha-amylase. It was concluded that, in response to GA, alpha-amylase was produced in the aleurone layer by *de novo* synthesis. However, in an intact system an inductive agent is not required, as, for example, in *de novo* synthesis of isocitritase in germinating peanut cotyledons as reported by Gientka-Rychter and Cherry (1968). They germinated intact peanut seeds in ^{14}C-reconstituted amino acids for 4 days and followed this by fractionation of a 20 to 35 percent ammonium sulfate preparation on a Sephadex G-200 column. The result showed that the active enzyme fraction coincided with a large peak of radioactivity. They combined these results with the result from the density-labeling method and concluded that isocitritase was synthesized *de novo* during seed germination. Data from this study demonstrated that the active enzyme region coincided with a peak of radioactivity in DEAE fraction. Therefore, the dipeptidase appears to be synthesized *de novo.*

Specific radioactivity of each step of enzyme purification. Specific radioactivity (SR) was used by Rotman and Spiegelman (1954) for determination of the *de novo* synthesis of beta-galactosidase in *E. coli.* They compared SR value (cpm/μg N) and specific enzyme activity (enzyme activity/min/μg N) of each step of enzyme purification and found that, as the purification of enzyme progressed, the SR values decreased. They suggested that the residual radioactivity was not enzymatic but rather was assignable to contaminating material.

However, nonenzymatic contamination with radioactivity may account for the transformation of ^{14}C-amino acids during the incubation. In this study SR values increased with each step of purification. This increase in SR value, which compared the amount of ^{14}C-incorporation into different proteins, also supports the *de novo* synthesis concept. Ashton and Dahmen (1967) showed by using vertical acrylamide gel electrophoresis and ultracentrifugation that fraction IV (DEAE fraction) contained only one protein. The gel electrophoresis pattern for the other fractions showed that there were at least three proteins in fraction III, four proteins in fraction II, and five proteins in fraction I.

Conclusions

These experiments provide strong evidence for *de novo* synthesis of this dipeptidase in squash cotyledons during germination, as suggested by the protein synthesis inhibitor studies of Sze and Ashton (1971). Further confirmatory evidence for *de novo* synthesis of this enzyme could be provided by demonstrating ^{14}C-amino acid incorporation throughout the polypeptide chain and utilizing the density-labeling technique for protein synthesis; however, these proofs are not essential.

GENERAL DISCUSSION

In squash seeds, reserve protein is one of the storage foods in cotyledons, and the presence of the embryo axis is required for the breakdown of this reserve material during germination (Wiley and Ashton, 1967). For complete hydrolysis the intact reserve protein is first attacked by proteinase, and this yields smaller peptides; then a series of peptidases become active and further decompose the peptides into amino acids. The entire sequence of metabolic steps of this hydrolysis has not been fully elucidated, but this study and previous findings provide important information for understanding the process. As suggested by Penner and Ashton (1967), both dipeptidase and proteinase are synthesized *de novo*. Comparing the time-course development of these two enzymes, it is apparent that their synthesis coincides with the process of hydrolysis. That is, proteinase development is initiated at an early stage of germination (the first day of germination), and dipeptidase is initiated somewhat later (the third day of germination). This sequential development of two enzymes may be controlled by their hormonal specificity and inhibitor interactions. Only BA can stimulate the proteinase activity, according to Penner and Ashton (1967); however, our data indicate that both BA and GA are able to enhance the dipeptidase synthesis. During the early stage of germination, seeds have inhibitors that inhibit certain metabolic reactions and enzyme synthesis. Since cytokinins are considered the most effective agent for counteracting the germination inhibitors (Khan, 1971), they may serve dual roles—that is, they

may promote protein synthesis as well as counteract the effect of inhibitors. However, mRNA is required for protein synthesis. It has been stated that the entire apparatus except mRNA necessary for protein synthesis is functional in the seeds (Marcus and Feeley, 1964). Messenger RNA is considered to be activated during the first 24-hour germination; prior to its activation, enzymes cannot be synthesized.

The catabolism of reserve protein is particularly important in seeds that have protein as their principal storage product, since storage protein not only supplies amino acids for protein synthesis but also provides energy for other metabolic reactions. Thus, herbicides that inhibit any of the enzymes in this system could prevent seed germination. The data presented previously in the section entitled "Effect of Herbicides on Dipeptidase Activity," which indicated several herbicides decreased the dipeptidase synthesis, when combined with information reported by Penner and Ashton (1968) show that inhibition of both dipeptidase and proteinase or severe inhibition of one of these enzymes was correlated with the inhibition of squash seed germination. The inhibition of dipeptidase (see previous section entitled "Influence of Herbicides on Hormonal Control of Dipeptidase") or proteinase (Penner and Ashton, 1968) induced by some herbicides was counteracted to a certain degree by the addition of certain hormones. However, the reversibility in enzyme activity did not result in the recovery of seed germination. This indicates that both hormones and herbicides affect more than one metabolic site, and therefore their antagonism may be different in each system.

SUMMARY

Effect of Herbicides on Dipeptidase Activity

The dipeptidase activity of the cotyledons of the squash (*Cucurbita maxima* Duch. 'Hubbard') increased during germination. Several herbicides inhibited this increase in various degrees. Those herbicides inhibiting the development of dipeptidase activity 70 percent or more were 2,4-D, dicamba, fenac, and naptalam. Those inhibiting dipeptidase activity by 63 to 34 percent were picloram, dichlobenil, bromoxynil, amitrole, atrazine, endothall, and bromacil. Those causing only slight inhibition, 24 to 16 percent, were diphenamid, chlorpropham, and trifluralin. Monuron was essentially the same as the control. However, dalapon, bensulide, CDAA, and CDEC were somewhat stimulatory. When compared with previously reported herbicide activity on proteinase activity, those herbicides that inhibited dipeptidase activity did not necessarily affect proteinase activity to the same degree.

Hormonal Regulation of Dipeptidase

Dipeptidase activity of cotyledons of squash increased during seed germination. The embryo axis controlled about 25 percent of this development for 4-

day germination. Four plant-growth regulators (GA, BA, IAA, and ABA), each with two concentrations, 5×10^{-6} M and 5×10^{-5} M, were examined for their effect on the dipeptidase activity. None of these regulators affected the activity *per se* of the isolated dipeptidase. The presence of growth regulators during the germination process caused various degrees of effects on the dipeptidase development. In intact seeds, BA and IAA inhibited dipeptidase activity at the higher concentration, 5×10^{-5} M; ABA reduced the activity at both concentrations, 5×10^{-6} M and 5×10^{-5} M; however, GA enhanced its development at the higher concentration, 5×10^{-5} M. In distal-half cotyledons, BA and GA both stimulated the enzyme development; however, their combination showed no additive or synergistic effect. ABA suppressed the dipeptidase activity at the higher concentration, and IAA showed an inhibition of the activity at both levels.

Influence of Herbicides on Hormonal Control of Dipeptidase

Five herbicides were examined for their influence on the dipeptidase activity and the growth of the distal-half cotyledons of squash during the incubation. None of these herbicides affected the enzymatic reaction *per se* of the isolated dipeptidase. The presence of the herbicides in the culture solution caused different effects on the growth and the development of dipeptidase activity. However, it is difficult to find any correlation between growth of distal-halves and the enzyme activity. Dicamba, 2,4-D, and naptalam showed no effect on growth but did reduce enzyme development significantly. The addition of BA or GA relieved a certain degree of inhibition produced by these three herbicides. Dichlobenil inhibited the dipeptidase activity very little; however, it stopped the growth of this tissue. Endothall damaged the distal-halves, but it promoted enzyme activity. Data on interaction of these two herbicides with GA and BA are inconclusive.

***De novo* Synthesis of Dipeptidase**

By using the radioactive amino acid incorporation method, this study provides strong evidence for the theory of *de novo* synthesis of this dipeptidase in squash cotyledons during germination, as suggested by Sze and Ashton (1971). This theory is based on the fact that the amount of ^{14}C-leucine incorporated into dipeptidase was greater than that found in the other proteins. The greater amount of ^{14}C-incorporation was demonstrated by (a) the action enzyme region coincided with a peak of radioactivity in the DEAE fraction, and (b) the specific radioactivity of the enzyme increased with each step of purification. The result also indicates that there is a positive agreement between the rate of ^{14}C-leucine incorporation into the dipeptidase and the rate of dipeptidase development.

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Chapter 2.
Effects of Herbicides on Bud Sprouting of Purple Nutsedge

R. K. Nishimoto

INTRODUCTION

The objectives of this study were (a) to determine the basis of bud dormancy in purple nutsedge— if dormancy can be overcome and sprouting initiated as desired, the effect of repeated herbicide applications on the sprouting ability of these dormant buds on tubers can be determined; and (b) to compare the effects of repeated foliar applications of glyphosate [*N*-(phosphonomethyl) glycine] and several other herbicides on the growth and reproduction of purple nutsedge.

Purple nutsedge *(Cyperus rotundus)* is the most troublesome weed of the tropical world, mainly because of its biological adaptability. This weed is a perennial, possessing an extensive vegetative structure of rhizomes and tubers that are also propagative; these tubers may remain dormant in the soil, and this further favors its survival. All these factors have made eradication of nutsedge difficult, and to date no economical control method is available.

Purple nutsedge appears tolerant to many herbicides primarily because more shoots will sprout if one is killed by herbicides. Varying levels of control have been achieved by the use of 2,4-D [(2,4-dichlorophenoxy)acetic acid], dicamba (3,6-dichloro-*o*-anisic acid), amitrole (3-amino-*s*-triazole), and MSMA (monosodium methanearsonate) (Hauser, 1963; Magalhaes et al., 1968; Hamilton, 1971). Recently, glyphosate was reported to reduce purple nutsedge stands in Tanzania (Magambo and Terry, 1973). Even if tuber infestation is reduced to a low level, purple nutsedge can quickly reinfest an area; it has been estimated that tubers planted at 30-cm spacing will produce 2.1 million plants and 4.4 million tubers per 0.4 ha in 1 year (Hauser, 1962).

LITERATURE REVIEW

The development of dormant tubers enables nutsedge to adapt to cropping methods of various cultures, thus making its eradication difficult. Black tubers exhibit the greatest degree of dormancy, while young white tubers sprout easily. Dormant tubers have from 1 to 13 buds (Ueki, 1969). Only one or two buds sprout under suitable conditions; the others remain dormant. Apical

buds inhibit sprouting of other buds on the same tuber (Muzik and Cruzado, 1953). Similarly, Jangaard et al. (1971) noted that upon the death of the growing plant's foliage, inhibition of tuber sprouting was relieved, and sprouting of other buds occurred. Dissecting the tubers also stimulated sprouting of dormant buds near the cut surface (Muzik and Cruzado, 1953).

An endogenous promoter-inhibitor complex is commonly believed to control dormancy in seeds and resting buds (Wareing and Saunders, 1971). The endogenous promoters may include gibberellins (GA) and cytokinins. Many inhibitors are postulated to be present, but those most often cited are abscisic acid (ABA) and phenolic compounds. The role of cytokinin in releasing dormancy, in some cases, is to antagonize action of an endogenous inhibitor. In the absence of endogenous inhibitors, cytokinin is dispensable. Fisher (1971) suggested that a high level of the endogenous inhibitor gibberellic acid, together with a deficiency of cytokinin, caused bud inhibition in *Cyperus alternifolius* L.

Bud dormancy in nutsedge is suspected by many to be due to the presence of inhibitors (Ueki, 1969). Washing tubers before sprouting increased the number of sprouts produced per tuber, suggesting that the inhibitor was water soluble. Berger and Day (1967) reported that salicylic acid was the major component among the many inhibitors found in the foliage of purple nutsedge; however, it was not found in the tubers. Jangaard et al. (1971) thought that ABA might be the inhibitor associated with nutsedge tuber dormancy, but they were unable to detect it in the dormant tubers (personal communication). Jackson et al. (1971) reported that ethylene and ethephon stimulated bud sprouting in nutsedge.

BUD SPROUTING OF PURPLE NUTSEDGE TUBERS

BA (*N*, 6-benzyladenine) at 50 and 300 ppm stimulated bud sprouting, producing two to three times as many sprouts per treatment as the controls (Table 2–1). In the controls, only one long apical sprout was usually formed. Increased BA concentrations inhibited root formation and decreased mean length of sprouts. None of the other growth regulators tested stimulated sprouting. Apparently, sprout stimulation occurred only when tubers were incubated continuously in a BA solution (Table 2–2). Higher temperatures produced more bud sprouts (Table 2–3); at the lower temperature regime, BA-stimulated sprouting occurred only at 50 ppm.

As suggested by data in Table 2–2, BA is apparently required as an agent for the sprouting process rather than being merely a trigger mechanism to release dormancy. Further, there is an implication that dormancy in nutsedge is due to a deficiency of this sprouting agent, causing an unfavorable balance in the promoter-inhibitor complex in the tuber.

Table 2-1. Effect of BA on purple nutsedge sprouting and sprout length

Treatment	Concentration (ppm)	Sprouts per treatment[1] (no./plant)	Sprout length[1] Maximum (mm)	Minimum (mm)	Mean (mm)
Water control		19.2 b	67 a	3	22
Ethanol control		18.0 b	72 a	3	22
BA	1	8.7 a	76 a	4	27
BA	10	23.9 b	74 a	2	20
BA	50	34.3 c	68 a	3	19
BA	300	48.4 d	32 b	6	14

[1]Means within a column bearing a different postscript letter are significantly different ($P<0.05$) (Duncan's Multiple Range test).

SOURCE: Teo et al., 1973.

Table 2-2. Effect of soaking purple nutsedge tubers in 100 ppm of BA for different lengths of time

Time (hours)	Sprouts per treatment[1] (no./plant)
0 (water control)	14.8 a
3	13.8 a
6	14.0 a
12	16.5 a
24	15.5 a
240 (BA control)[2]	29.0 b

[1]Means within a column bearing a different postscript letter are significantly different ($P<0.05$) (Duncan's Multiple Range test).
[2]Tubers remained in BA solution during the 10 days of the study.

SOURCE: Teo et al., 1973.

On the other hand, ABA inhibited sprouting at higher concentrations (Table 2-4). When tubers were pretreated with BA and later incubated in ABA, sprouting was also inhibited, but to a lesser degree. Further exposure to BA stimulated sprouting. These data suggest that dormancy in nutsedge tubers may be due to a hormonal imbalance, favoring such substances as ABA. BA is the suggested promoter in this system.

Growth of plants from tubers pretreated with 100 ppm BA did not differ significantly from untreated tubers when measuring parameters such as flowering time, number of inflorescences, and number and total length of rhizomes after 5½ weeks (Table 2-5). However, continuous BA applications induced a tuft-type growth, with short, dark green leaves. Flowering was delayed, and the total number of shoots produced from an initial plant in-

creased. BA induced production of numerous short, diageotropic rhizomes. In untreated plants, rhizomes were branched, whereas no branching was observed with BA treatment.

BA, as well as PBA [6-benzylamino-9 (tetrahydropyran-2-yl)-9H-purine] also induced sprouting of tubers in soil when applied as a drench. From 70 to 90 percent of the buds on the tubers sprouted when grown in soil with either

Table 2-3. Effect of temperature on number of purple nutsedge sprouts

		Sprouts per treatment[1]	
Treatment	Concentration (ppm)	33°C day; 25°C night (no./plant)	24°C day; 17°C night (no./plant)
Water control		23.6 c	6.0 a
Ethanol control		22.8 c	3.8 a
BA	1	9.2 b	5.8 a
BA	10	20.6 c	5.0 a
BA	50	31.4 d	13.0 b
BA	300	46.4 e	6.0 a

[1]Means within a column bearing a different postscript letter are significantly different ($P<0.05$) (Duncan's Multiple Range test).

SOURCE: Teo et al., 1973.

Table 2-4. Effect of ABA and combinations of BA and ABA on purple nutsedge sprouting

		Sprouts per treatment (no./plant)[1]			
Treatment	Concentration (ppm)	ABA	BA + ABA[2]	BA + ABA→BA[3]	Increase
BA control[4]	100		22.3 a	22.3 a	
Water control		10.3 a			
ABA	1	13.5 a	18.5 a	22.3 a	3.8
ABA	10	8.6 a	21.8 a	24.8 a	3.0
ABA	100	0.3 b	8.3 b	15.5 a	7.2

[1]Means within a column bearing a different postscript letter are significantly different ($P<0.05$) (Duncan's Multiple Range test).

[2]Tubers soaked in BA for 24 hours, followed by 6 hours in ABA, then allowed to sprout for 10 days on moistened filter paper.

[3]After 10 days, the BA + ABA-treated tubers were exposed to BA to induce remaining buds to sprout.

[4]Tubers remained in BA solution throughout the study.

SOURCE: Teo et al., 1973.

Table 2-5. Effect of continuous foliar application of BA on purple nutsedge growth

BA concentration (ppm)	Growth of initial plants[1]				
	Shoots (no./plant)	Inflorescences (no./plant)	Rhizomes (no./plant)	Length (mm)	Mean length of primary rhizomes (mm)
0	11.2 ab	4.2 a	19.1 ab	1346 a	47 a
10	9.0 a	2.6 a	14.9 a	861 a	66 a
100	17.7 c	0.2 b	36.6 c	1469 a	21 b
200	19.4 c	0.5 b	34.9 c	1115 a	24 b

[1]Means within a column bearing a different postscript letter are significantly different ($P<0.05$) (Duncan's Multiple Range test).

SOURCE: Teo et al., 1973.

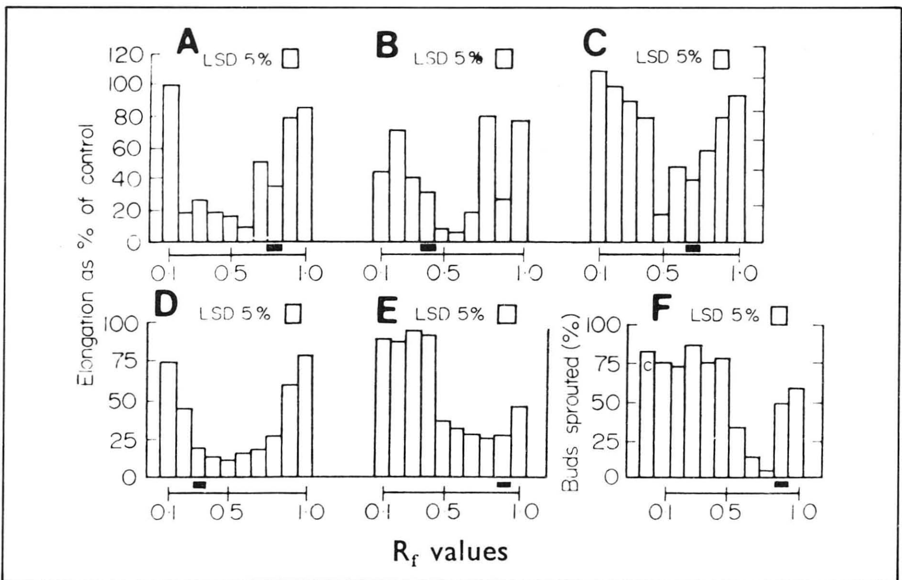

Figure 2-1. Bioassay of activity of acidic ether extract from 15 g *C. rotundus* tubers (A, B, C) and the various components of inhibitor β from 10 g *C. rotundus* tubers (D, E), using the wheat coleoptile assay and, with the addition of 1 ppm BA, using the *C. rotundus* excised bud assay (F). Extracts were run on TLC and developed with the following solvent systems: A—butanol:acetic acid:water (5:4:1); B—chloroform:ethyl acetate:acetic acid (60:40:5); C—isopropanol:ammonium hydroxide:water (100:14:6); D—chloroform:acetic acid (95:5); and E, F—ether:ethyl acetate:acetic acid (50:5:2). The C of histogram F indicates silica gel control. The position of marker ABA is shown by a solid rectangle on the X axis. From Teo et al., 1974.

BA or PBA, while only 25 percent of the buds sprouted in the control. Furthermore, BA activity in the soil lasted for less than 1 week.

When inhibitors were extracted from tubers, only the acidic ether extract showed activity, and this fraction was further investigated. TLC (thin layer chromatography) separation of the extract indicated that more than one inhibitory substance was present in nutsedge tubers (Figure 2–1), and, based on the similarity of the Rf values in the solvent system used (isopropanol:ammonium hydroxide:water [100:14:6] hereafter referred to as PAW), the inhibitors were collectively referred to as the inhibitor *β* complex of earlier workers (Bennet-Clark and Kefford, 1953; Hemberg, 1958; Szalai, 1959; Holst, 1971; Wareing and Saunders, 1971). Rechromatography and bioassay of this *β* inhibitor complex tested by the excised nutsedge bud assay (Figure 2–1, F) gave broadly similar results to the wheat coleoptile assay (Figure 2–1, E). Five zones (Rf 0.6 to 1.0) inhibited bud sprouting, and the marker ABA corresponded to Rf 0.9 (Figure 2–1, F).

Figure 2–2 shows a comparison of the activity of ABA and the inhibitors from nutsedge tubers. Fifty percent inhibition of wheat coleoptile elongation was obtained with the inhibitor *β* complex from 3 g of tubers (Figure 2–2, A, with ABA at 0.5 ppm (Figure 2–2, D), and with the Rf 0.9 component from 6 g

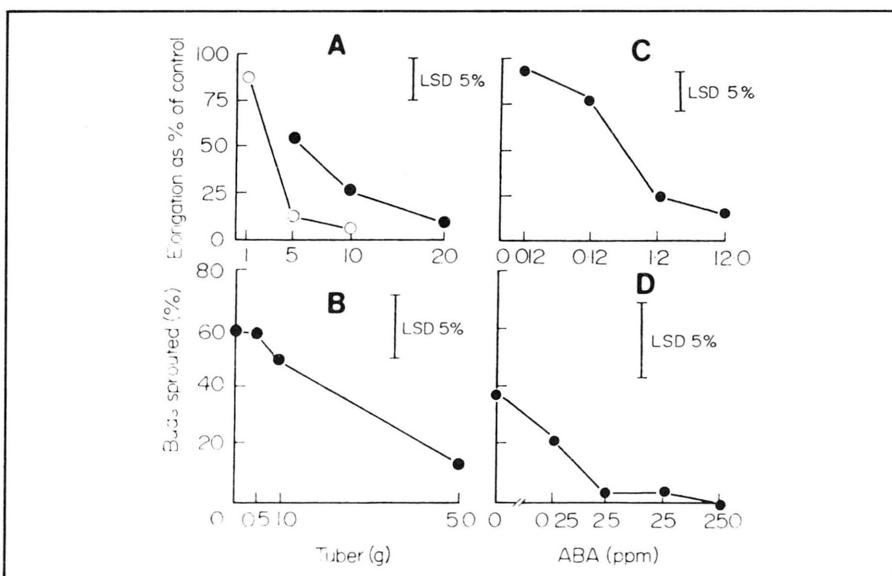

Figure 2–2. Activity of inhibitors from *C. rotundus* tubers and ABA. A—inhibitor *β* complex; B—Rf 0.9 component of inhibitor *β* complex further chromatographed with ether:ethyl acetate:acetic acid (50:5:2); and C, D—ABA. A and C were bioassayed with the wheat coleoptile test, and B and D with excised buds of *C. rotundus* but without the addition of BA. From Teo et al., 1974.

of tubers (Figure 2–2, A). Fifty percent inhibition of bud sprouting was obtained with Rf 0.9 component from 3 g of tubers (Figure 2–2, B) and with ABA at 0.5 ppm (Figure 2–2, D).

Inhibitor *β* from PAW significantly inhibited excised bud sprouting partially or completely, depending on the weight of tubers extracted. The addition of 100 ppm BA to the extract relieved partial sprout inhibition but was ineffective on completely inhibited buds.

Bud-sprouting inhibition induced by the various components of inhibitor *β* was also relieved by treatment with BA; thus none of the components caused complete inhibition. Inhibitors extracted from 10 g of tubers inhibited bud sprouting even with BA present. One hundred ppm BA did not reverse bud-sprouting inhibition, except for the inhibitors present at Rf 0.9, which should have included any endogenous ABA (Table 2–6). Bud sprouting was severely inhibited by exogenous ABA, and this inhibition was reversed with varying amounts of BA application. Inhibition of buds by 250 ppm ABA was not reversed by 100 ppm BA.

The gas liquid chromatography (GLC) analysis of the BSA [*N*, O-Bis (trimethylsilyl) acetamide] treated inhibitor *β* complex indicated the presence of a component having an identical retention time (20 min) to the standard trimethylsilyl derivative of ABA (TMS-ABA). Upon further TLC purification of the inhibitor *β* complex in ether:ethyl acetate:acetic acid (50:5:2), the materials eluted from Rf 0.9 of the chromatogram were analyzed alone and with added ABA after silylation. The ABA added to the eluate was coincident with a peak in the unfortified eluate (Figure 2–3). GLC analysis of the other inhibitory zones (Rf 0.6, 0.7, and 0.8) suggested that they did not contain any ABA-like substance. This evidence, supported by the bioassays of the eluates,

Table 2–6. Effect of components from the *β* inhibitor complex from purple nutsedge tubers rechromatographed in ether:ethyl acetate:acetic acid (50:5:2), and of BA on sprouting of excised buds of purple nutsedge tubers

Rf in second solvent	Percentage of buds sprouted[1]			
	5 g tubers	5 g tubers + 1 ppm BA	10 g tubers + 1 ppm BA	10 g tubers + 100 ppm BA
Water control	72 ad	94 a	84 ad	90 a
Rf 0.6	30 b	66 ad	34 bc	10 b
Rf 0.7	26 b	66 ad	13 b	0 e
Rf 0.8	15 b	86 ad	5 b	0 e
Rf 0.9	10 b	54 cd	50 c	80 ad

[1]Means bearing a different postscript letter are significantly different ($P<0.05$) (Duncan's Multiple Range test).

SOURCE: Teo et al., 1974.

strongly indicates that ABA is one of the minor components of inhibitor *β* complex.

Spraying a strip of a TLC plate of the inhibitor *β* complex with diazotised *p*-nitro-aniline (DPNA) produced a deep yellow coloration between Rf 0.5 and 0.8, suggesting that some of these substances were phenolics. A similar chromatogram strip sprayed with a sucrose-HCl-ethanol reagent showed a pink-violet color at Rf 0.6 and violet at Rf 0.8, thus indicating that these might be di- or trihydroxy phenols. A light gray spot obtained at Rf 0.7 when a strip was treated with 2 percent aqueous ferric chloride was again indicative of a phenolic compound.

Although this work established the presence of the inhibitor *β* complex (including ABA and phenols) in purple nutsedge tubers, this is not sufficient evidence to substantiate its possible physiological role in causing tuber dormancy. The results suggest a hypothesis implicating a possible relationship between inhibitor *β* or ABA and bud dormancy of tubers in this species. BA reversal of the inhibitory effects caused by the inhibitor *β* complex and ABA

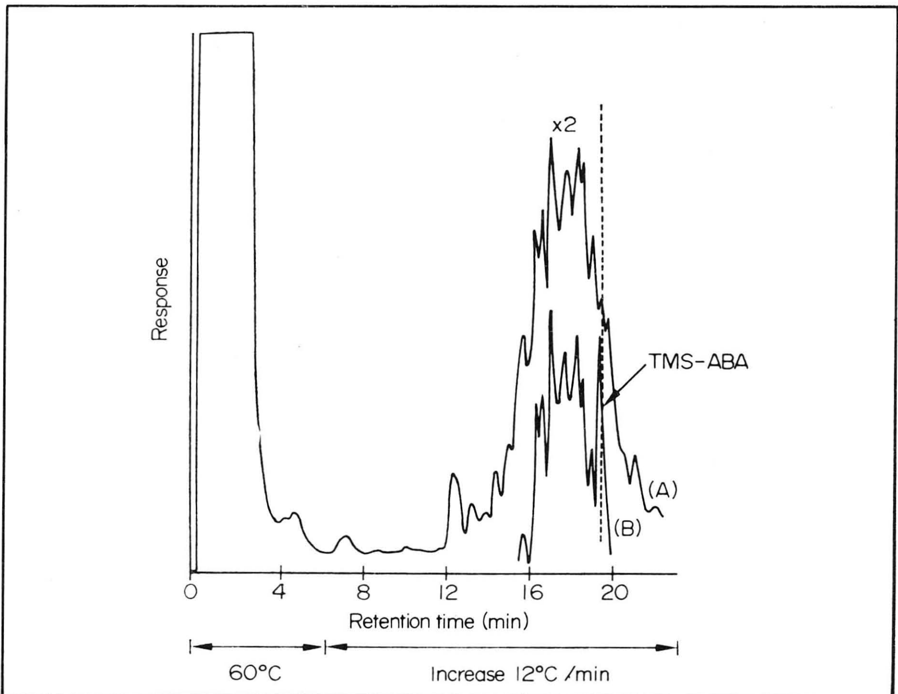

Figure 2–3. Gas chromatograms of a silylated eluate from Rf 0.9 component of inhibitor *β* complex of *C. rotundus* tubers rechromatographed in ether:ethyl acetate:acetic acid (50:5:2), without (A) or with (B) the addition of standard ABA. From Teo et al., 1974.

supports the hypothesis that a balance between inhibitors and promoters controls dormancy in nutsedge tubers. Treatment of buds with inhibitor β or ABA shifts the balance toward sprouting inhibition; addition of BA to the sprouting media permits sprouting. In this hypothesis, the role of BA in releasing dormancy is to antagonize the endogenous inhibitor action.

This hypothesis explaining dormancy in purple nutsedge suggests that dormant tubers are deficient in a cytokinin, and this leads to an imbalance in the promoter-inhibitor complex favoring the inhibition. ABA and the other components of the inhibitor β complex are likely natural inhibitors. Accumulation of a cytokinin, or disappearance of the inhibitors, would be necessary to restore a favorable balance of growth promoters, allowing sprouting to occur. The effect of exogenous BA is to counteract the inhibitor action in a manner similar to that postulated for the action of a cytokinin.

RESPONSE OF PURPLE NUTSEDGE TO REPEATED APPLICATIONS OF HERBICIDES

Glyphosate reduced purple nutsedge stands in the field (Table 2-7). The second and third glyphosate treatments reduced the stands substantially but did not completely eliminate the population. The importance of complete elimination in an infested field was clearly demonstrated in this experiment (Table 2-7). When the field was left undisturbed for 10 weeks after the third treatment, the nutsedge population increased to about 35 and 19 percent of the control, which demonstrates the re-establishment potential of uncontrolled nutsedge.

Table 2-7. Effect of three consecutive herbicide treatments on purple nutsedge stand

		Number of plants (no./0.1 m^2)			
Herbicide	Rate (kg/ha)	4 weeks after first treatment	4 weeks after second treatment	4 weeks after third treatment	10 weeks after third treatment
Glyphosate	2	32.8	2.8	2.3	13.8
Glyphosate	4	14.5	1.8	1.3	7.5
MSMA	2	35.0	13.8	22.8	22.8
Paraquat	1	31.5	40.3	37.3	50.3
Dicamba	1	40.3	23.5	54.3	32.8
Control		44.3	47.3	66.3	39.0
LSD* 0.05		ns	24.0	45.8	22.0

*Least significant difference.

SOURCE: Zandstra et al., 1974.

MSMA significantly reduced the nutsedge stand 4 weeks after the second treatment; at other times, however, it did not differ from the control. Paraquat (1,1-dimethyl-4,4' bipyridinium ion) and dicamba had no effect on the nutsedge stands.

After the field was rotovated (working the soil thoroughly to a depth of 15 cm) on completion of the first stage of the experiment, the initial nutsedge stand did not differ between treatments (Table 2-8). This similarity may be due to several factors. Rotovating the soil may have raised dormant tubers from the lower soil levels, which had not been under the influence of the herbicide treatments. Since the field was left untreated for 10 weeks before rotovating, the nutsedge may have re-established itself in the treated plots. This allowed for the production of new reproductive structures, and these structures sprouted after the field was rotovated.

The reduction of the nutsedge stand due to glyphosate treatments in the second stage of the experiment (Table 2-8) was similar to that described earlier (Table 2-7). Likewise, paraquat and dicamba had no effect on the stand. Repeated applications of MSMA reduced it to a level similar to that of the glyphosate treatments. This result supports earlier observations of Hamilton (1971) that repeated applications of MSMA are necessary to control nutsedge.

In addition to destroying the leaves and reducing the plant population, the effect of repeated applications of glyphosate and MSMA was to reduce tuber production (Table 2-9). Treatments with glyphosate and MSMA reduced tuber production by 92 and 88 percent of the control, respectively. Paraquat and dicamba did not reduce tuber production.

The number of tubers that sprouted after treatment with glyphosate and MSMA was reduced when compared with the control (Table 2-9). These data

Table 2-8. Effect of two consecutive herbicide treatments on purple nutsedge stand after field was rotovated

Herbicide	Rate (kg/ha)	Number of plants (no./0.1 m²) Initial stand	6 weeks after first treatment	6 weeks after second treatment
Glyphosate	2	10.5	3.8	7.5
Glyphosate	4	10.0	3.3	6.0
MSMA	2	8.8	7.5	7.3
Paraquat	1	12.5	18.3	27.5
Dicamba	1	12.8	17.5	23.8
Control		11.8	32.0	51.3
LSD* 0.05		ns	17.0	17.8

*Least significant difference.

SOURCE: Zanstra et al., 1974.

Table 2-9. Production and viability of purple nutsedge tubers after repeated herbicide treatment

Herbicide	Rate (kg/ha)	Tubers present[1] (no./0.1 m²)	Tubers sprouted with BA treatment[2] (%)	Sprouts per viable tuber (no./plant)
Glyphosate	2	14.0	30	4.5
Glyphosate	4	14.5	33	5.2
MSMA	2	20.0	27	3.6
Paraquat	1	77.5	85	3.2
Dicamba	1	82.0	57	3.5
Control		172.0	82	4.4
LSD* 0.05		111.0	20	ns

[1]Tubers obtained from the upper 13 cm of soil.
[2]Ten tubers per petri dish sprouted with 100 ppm BA.
*Least significant difference.

SOURCE: Zandstra et al., 1974.

seem to indicate that foliar applications of glyphosate and MSMA also affected tuber viability, as measured by BA-induced sprouting. Tubers whose viability was not affected produced as many sprouts as the control (Table 2-9), indicating that buds on these sprouted tubers had not accumulated toxic levels of the herbicides.

In the greenhouse, glyphosate reduced the fresh weight of green leaves of purple nutsedge and the number of sprouts produced from the original tubers, new tubers, and basal bulbs (Table 2-10). Visual examination of nonsprouting tubers indicated that some of their tissues were necrotic, suggesting that glyphosate was translocated and affected the viability of original and new-developing tubers.

Differential translocation of glyphosate could explain why some tubers were killed and others were not. An established purple nutsedge stand consists

Table 2-10. Fresh weight of green leaves, viability of original tubers, and viability of new tubers 4 weeks after treatment with glyphosate at 4 kg/ha

Treatment	Fresh weight of green leaves[1] (g)	Sprouts per tuber planted[1] (no./plant)	Sprouts per new tuber and basal bulbs[1,2] (no./plant)
Control	3.35	0.93	5.09
Glyphosate	0.31**	0.12**	0.16**

[1]Values marked (**) were significantly different from the controls at the 1% level.
[2]Structures produced from the original tubers.

SOURCE: Zandstra et al., 1974.

of a complex interconnected system of plants, rhizomes, basal bulbs, and tubers (Ranade and Burns, 1925). Plants may arise directly from the tuber or from rhizomes at varying distances from the tuber. On the basis of distance, different degrees of translocation of foliarly applied herbicides to underground structures could be expected. Also, some tubers were probably already dormant at the time of application, and less translocation to these tubers would be expected.

SUMMARY

Various concentrations of BA were used to induce sprouting of dormant purple nutsedge tubers. BA at 50 to 300 ppm stimulated sprouting. The continuous presence of BA during the sprouting period was necessary to give significant sprout stimulation. ABA counteracted the stimulatory effects of BA when tubers were treated with ABA after BA treatment. Sprouting was markedly greater with days at 33°C and nights at 25°C than with days at 24°C and nights at 17°C. Growth of plants originating from tubers pretreated with 100 ppm BA did not differ significantly from the controls. Sustained BA applications at 100 and 200 ppm produced numerous plants with tuft-type growth habit, delayed flowering, and reduced the number of inflorescences. Numerous short, diageotropic rhizomes were produced.

BA and PBA stimulated bud sprouting of purple nutsedge in soil. BA activity in soil was less than 1 week.

The acidic ether fraction of methanol extracts from *C. rotundus* tubers contained inhibitory substances described as inhibitor β. Inhibitor β inhibited sprouting of excised nutsedge buds and elongation of wheat coleoptiles. Bud-sprouting inhibition by inhibitor β was reversed by the application of BA. Applications of ABA also inhibited sprouting of excised buds, and this was similarly reversed by BA applications. Chromatographic evidence suggested that inhibitor β consisted mainly of phenolic materials and possibly ABA as a minor component.

Purple nutsedge plants were treated in both the greenhouse and the field with glyphosate. In the field, glyphosate treatments at 2 and 4 kg/ha were compared with several herbicides for nutsedge control in repeated applications over an 8-month period. After several applications, glyphosate reduced the number of plants per unit area. After rotovation and reapplication, glyphosate and MSMA plots had fewer plants and tubers per unit area than controls. These tubers were also less viable than tubers from control plots. In the greenhouse, fresh weight of leaves, sprouts per original tuber, and sprouts per new tuber were reduced by 4 kg/ha glyphosate.

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Chapter 3. Action of Sandoz 6706 on Carotenoid Synthesis and Chloroplasts

P. G. Bartels

INTRODUCTION AND LITERATURE REVIEW

Sandoz 6706 [4-chloro-5-(dimethylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3 (2H)-pyridazinone] is an experimental herbicide that shows promise for selective preemergence control of many broadleaf and grassy weeds, including nutsedge (*Cyperus* sp.), in cotton fields. Cotton plants absorb and retain Sandoz 6706 in their roots but translocate less of the herbicide to their shoots than do corn and soybean plants. This differential translocation of herbicide could explain the differences in the tolerance of cotton (resistant), corn (very susceptible), and soybean (susceptible) to Sandoz 6706 (Strang and Rogers, 1974).

The chemical structure of Sandoz 6706 is similar to that of the herbicide pyrazon [5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone]. The initial visible symptom of Sandoz 6706 toxicity to plants, however, is unlike that of pyrazon but is similar to symptoms caused by the herbicides amitrole and dichlormate. The ultrastructural study by Anderson and Schaelling (1970) revealed that the earliest symptom of pyrazon injury is swelling of the chloroplast in mature bean leaves; no immediate bleaching of the leaf tissue was observed after pyrazon treatment. Anderson and Schaelling (1970) also reported a marked increase in the number and size of osmiophilic plastoglobuli in the chloroplast. Accumulation of plastoglobuli may result from an inhibition of thylakoid formation. In contrast, Sandoz 6706 has a pronounced effect on the chloroplast pigments. New foliage formed in the light after treatment with sublethal application of Sandoz 6706 is either a bleached white or anthocyanin red, as determined by the plant species' capacities for anthocyanin production. Sandoz 6706 does not inhibit seed germination or normal leaf emergence.

Hilton et al. (1969) studied the effect of Sandoz 6706 on established green leaves and developing tissue of several different species. They found that the Hill reaction and CO_2 fixation were inhibited in older established leaves, whereas lipid uptake and utilization were affected in young growing tissue. They suggested that Sandoz 6706 might act as a direct inhibitor of an early

stage of biosynthesis of isoprenoid lipids from acetate. This herbicide may also inhibit utilization of lipids necessary for formation of lamellar membranes or other structural components of the chloroplast. Hilton et al. (1971) reported that Sandoz 6706 reduced the amount of fatty acid found in polar lipids (galactolipids) of chloroplasts and increased the amount in nonpolar lipids, while having little effect on the total content of bound fatty acids. Also, Sandoz 6706 reduced the amount of linolenic acid relative to linoleic acid in leaves and chloroplasts. D-α-tocopherol acetate, phytol, farnesol, squalene, unsaturated fatty acids, and fatty acid methyl esters could circumvent some of these inhibitory effects of Sandoz 6706, especially on plastid pigment accumulation and fatty acid buildup. It was noted that trifluoromethyl substitution on the phenyl ring of Sandoz 6706 was necessary for its herbicidal activity (Hilton et al., 1969).

FUNCTION OF CAROTENOID IN PLANTS

Carotenoids protect cells of photosynthetic bacteria from lethal photo-induced reactions. In experiments with bacterial cells lacking normal carotenoids, bacteriochlorophyll was bleached and cells were destroyed when they were illuminated under aerobic conditions; normal carotenoids prevented bleaching and photodestruction of cells (Cohen-Bazire and Stanier, 1958; Fuller and Anderson, 1958). Carotenoids also protect cells of nonphotosynthetic bacteria from damage due to photo-oxidation catalyzed by other light-absorbing pigments (Mathews, 1964).

Kohl (1902) was first to suggest that carotenoids might protect chlorophyll. The protective action is now believed due to carotenoids with nine conjugated double bonds or more quenching excited states of chlorophyll (Fujimori and Livingston, 1957). Krinsky (1967) reported that carotenoids could remove oxygen from excited chlorophyll-oxygen complexes via a carotenoid-epoxide cycle. This would prevent photo-oxidation or bleaching of the chlorophyll, and could explain the protective action of carotenoids.

There is much evidence extending the protective theory of carotenoids to higher plants. Koski and Smith (1951) found that a bleached mutant of corn was actually a carotenoid-less mutant. This mutant synthesized protochlorophyll(ide) in the dark and converted it to chlorophyll(ide) upon illumination. With continuous illumination under aerobic conditions, however, whatever chlorophyll the mutant synthesized was lost. Anderson and Robertson (1960) showed this mutant to have a block between the carotenoid precursor phytoene and normal carotenoids. Walles (1967) found that a sunflower mutant that did not have normal carotenoid pigments contained disrupted plastids. This mutant formed normal etioplasts when grown in the dark. When the mutant was exposed to light, thylakoid and vesicles dispersed normally

throughout the plastid, but continued illumination resulted in plastid disruption and bleaching of the sunflower seedlings. Faludi-Daniel et al. (1965) found two other photosensitive mutants of corn with blocks in the carotenoid-synthesizing system. All these carotenoid-free mutants accumulated chlorophyll under dim light but were bleached when exposed to bright light.

Various mechanisms of action for herbicides have been proposed by different researchers (Ashton and Crafts, 1973). However, only recently has the inhibition of carotenogenesis been considered a possible mechanism of action of several chemically unrelated herbicides (Burns et al., 1971). Burns et al. (1971) have shown that treatment of wheat seedlings with amitrole (3-amino-*s*-triazole), dichlormate (3,4-dichlorobenzyl methylcarbamate), and pyriclor (2,3,5-tricloro-4-pyridinol) resulted in inhibition of the normal biosynthetic pathway of carotenoids and, as a consequence, in photodestruction of chlorophyll and disruption of chloroplast. Their work showed that ζ-carotene accumulated in the dichlormate-treated plants, whereas phytoene, phytofluene, and ζ-carotene accumulated in amitrole- and pyriclor-treated plants.

CAROTENOGENESIS: INHIBITION BY SANDOZ 6706

Biosynthetic Pathway

Light- and dark-grown wheat seedlings synthesize and accumulate carotenoid in their plastids. Goodwin (1971) proposed that these pigments were biosynthesized from mevalonic acid and isopentenyl pyrophosphate via geranyl geranyl pyrophosphate, two molecules of which condense to form phytoene. In a series of dehydrogenation reactions, phytoene is changed to carotenoid containing fully conjugated double bonds. Cyclization of these carotenoids occurs next. Specifically, carotenogenesis is believed to proceed as follows: phytoene (three conjugated double bonds) → phytofluene (five) → ζ-carotene (seven) → neurosporene (nine) → carotenes and xanthophylls (eleven) (Goodwin, 1971).

Carotenoids of Control Wheat Seedlings

Our research shows that carotene extracts from untreated wheat seedlings have absorption maxima of 477, 450, and 427 nm (Figure 3–1), which are characteristic of β-carotene. The β-carotene concentrations ranged from 13 μg/g fresh weight (FW) in dark-grown plants to 26 μg/g FW in 1500 ft-c-grown control plants (Table 3–1). In the ultraviolet (UV) part of the spectrum (Figure 3–1), absorption maxima of 264 and 260 nm were observed. The compound responsible for these absorbances could not be identified, but the maxima observed are not those of phytoene (Davis, 1965). The concentration of xanthophylls ranged from 53 to 90 μg/g FW (Table 3–1). When the carotenoids of

Table 3-1. Concentrations of carotenoids and precursors in Sandoz 6706-treated and control wheat seedlings, grown under 16-hour photoperiod at 21 °C. The 6706 concentrations used were 10^{-4} and 10^{-6} M[1].

Growth conditions 6-day-old seedlings	Xanthophyll	β-carotene	Phytofluene (μg/g fresh weight)	Phytoene
Dark				
Sandoz 6706				
10^{-4}	0.3 ± 0.03	0.1 ± 0.03	2.4 ± 0.1	43.0 ± 5.0
10^{-6}	—[2]	6.0 ± 1.0	8.0 ± 1.5	33.0 ± 4.0
Control	53.0 ± 6.0	13.0 ± 2.0	none	none
1 ft-c				
Sandoz 6706				
10^{-4}	0.45 ± 0.03	0.4 ± 0.3	3.0 ± 0.2	61.0 ± 3.0
10^{-6}	—	8.0 ± 1.0	12.0 ± 2.0	38.0 ± 2.0
Control	55.0 ± 4.0	20.0 ± 2.0	none	none
1500 ft-c				
Sandoz 6706				
10^{-4}	0.2 ± 0.04	0.3 ± 0.04	0.7 ± 0.2	10.0 ± 2.0
Control	90.0 ± 6.0	26.0 ± 2.0	none	none

[1]Each value is an average of six determinations.
[2]No determinations were made.

control dark- and light-grown plants were subjected to thin-layer chromatography (TLC) on silica gel G, four carotenoid pigments were separated and identified as β-carotene, lutein, violoxanthin, and neoxanthin.

Carotenoids of Sandoz 6706-Treated Seedlings

The spectral analysis (Figure 3-1) of the carotene fraction from either dark, 1 ft-c, or 1500 ft-c Sandoz 6706-treated seedlings (10^{-4} or 10^{-5} M) showed that the spectra were identical and that the colored pigments (400–500 nm) were absent. Sandoz 6706 caused the accumulation of two colorless substances. These were judged to be phytoene and phytofluene on the basis of absorption maxima at 298, 285, and 275 nm and 367, 348, and 331 nm, respectively (Davis, 1965). The concentration of phytoene was 43 μg/g FW for dark-grown seedlings and 61 μg/g FW for 1 ft-c-grown seedlings (Table 3-1); there was also a slight accumulation of phytofluene (3.0 μg/g FW) in these seedlings. The reduced concentration (10 μg/g FW) of phytoene in plants grown under 1500 ft-c was probably caused by the photodestruction of phytoene. TLC of this extract showed no colored carotenoid pigments, but a fluorescent band was observed under UV light. This fluorescent band contained phytoene and phytofluene (as judged by the absorption spectra). In addition to these substances, Ben-Aziz and Koren (1974) found epoxy phytoene in treated wheat seedlings.

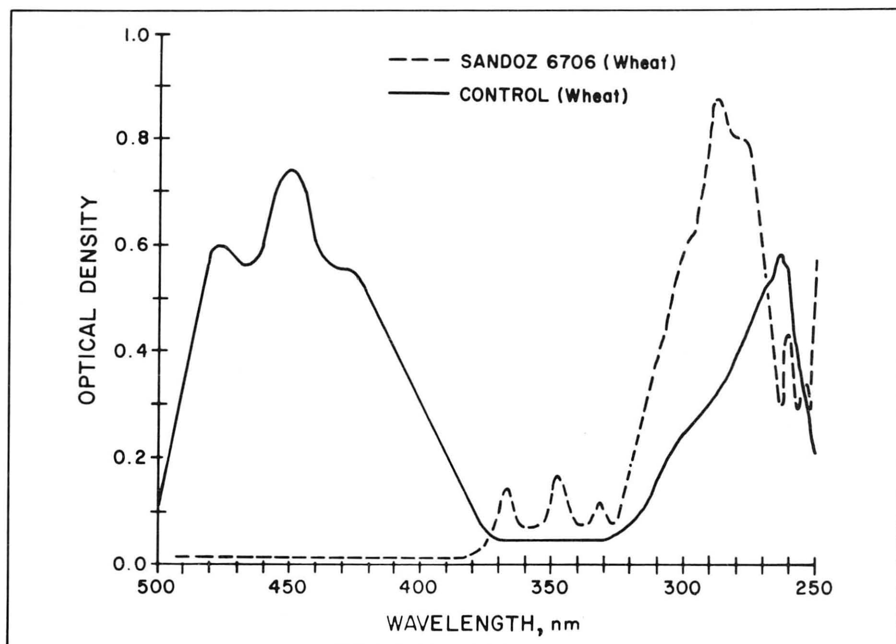

Figure 3-1. Absorption spectra of the carotene fraction (hexane) from control and Sandoz 6706-treated wheat seedlings grown either in the dark or at 1 ft-c or 1500 ft-c of light. Sandoz 6706 concentration was 10^{-4} M.

Seedlings treated with 10^{-6} M Sandoz 6706 had β-carotene as well as phytoene and phytofluene. The concentration of β-carotene in these seedlings was about 7 μg/g (Table 3-1), which was less than that found in control seedlings. Seedlings treated with 10^{-7} and 10^{-8} M Sandoz 6706 did not contain detectable quantities of phytoene and phytofluene, but had normal amounts of carotenoids. These results indicate that 10^{-4} and 10^{-5} M 6706 were most effective in inhibiting β-carotene biosynthesis while not affecting the growth of the seedlings.

Tocopherol and Chlorophyll

Table 3-2 shows that both treated and control seedlings grown at 1 ft-c contained α-tocopherol and chlorophyll. The concentrations of each in the 6706-treated seedlings were about 82 and 60 percent, respectively, of those found in the control seedlings. The chlorophyll of these treated plants contained the phytol ester. In dark-grown seedlings, 6706-treated seedlings had about 79 percent as much α-tocopherol as the control. In treated seedlings grown at 1500 ft-c, both chlorophyll and α-tocopherol were drastically reduced. Since carotenoid pigments are absent in these leaves, photodestruction of the

Table 3-2. Concentrations of α-tocopherol and chlorophyll in Sandoz 6706-treated and control seedlings, grown under 16-hour photoperiod at 21 °C. The concentration of Sandoz 6706 was 10^{-4} M[1].

Growth conditions 6-day-old seedlings	α-tocopherol		Chlorophyll	
	Sandoz 6706	Control	Sandoz 6706	Control
	(μg/3 g fresh weight)			
Dark	18 ± 2	23 ± 3	Trace	Trace
1 ft-c	21 ± 2	25 ± 2	101 ± 7	178 ± 8
1500 ft-c	10 ± 0.6	37 ± 5	15 ± 5	798 ± 12

[1]Each value is an average of six determinations.

chlorophyll and α-tocopherol probably occurred. Anderson and Robertson (1960) suggested that carotenoids act as "chemical buffers" to protect chlorophyll from photo-oxidation. They reported that a carotenoid-less albino mutant of corn (white-3) produced chlorophyll when grown in dim light (0.5 ft-c); however, exposure of this mutant to bright light in the presence of air resulted in destruction of chlorophyll.

GENERAL DISCUSSION OF MODE OF ACTION OF SANDOZ 6706

Our research shows that Sandoz 6706 caused loss of carotenes and xanthophylls as well as accumulation of phytoene and phytofluene. To explain these effects, it is suggested that 6706 acts both as an inhibitor of dehydrogenation reactions leading from phytoene to unsaturated acyclic carotenoids and as a stimulator of phytoene formation. The action of 6706 in stimulating phytoene formation may not be direct. Presence in the control seedlings of the end product β-carotene might depress the synthesis of phytoene. In treated seedlings where the β-carotene concentration is nil, a release of feedback inhibition might occur. If the cyclization reaction were inhibited, an accumulation of colored acyclic carotenoids such as lycopene and ζ-carotene might be expected. Figure 3-1 shows that this did not occur; ζ-carotene has been shown to accumulate in wheat seedlings treated with dichlormate, amitrole, and pyriclor (Burns et al., 1971). The absence of cyclic carotenoids in 6706-treated seedlings is probably due to an inhibition of dehydrogenation reactions occurring before cyclization. Ben-Aziz and Koren (1974) reported that ζ-carotene accumulates as well as phytofluene and phytoene in 6706-treated wheat seedlings; they suggest that the herbicide acts as a cyclization inhibitor in carotenogenesis rather than on just the dehydrogenation steps of the pathway. The difference between our results and those of Ben-Aziz and Koren (1974) may be

explained by the fact that the wheat seedlings were treated and grown in a different manner. Our seedlings were germinated and grown in petri dishes containing solutions of Sandoz 6706 or distilled water, while Ben-Aziz and Koren (1974) incorporated 6706 into the soil. The soil may alter the herbicide, and/or the concentration of herbicide reaching the seedlings may vary. They found that chlorophyll and carotenoids accumulated for the first 7 days and then declined. In our work, no colored carotenoid pigments were accumulated at any time.

Another hypothesis, proposed by Hilton et al. (1971), suggests that the primary action of 6706 may not be restricted to carotenoid pathways *per se*, but rather the absence of carotenoids may be only one of several consequences of an inhibition affecting other chloroplast lipid constituents. These authors suggest that Sandoz 6706 might act as a direct inhibitor of an early stage of biosynthesis of isoprenoid lipids. Our results, however, do not support this hypothesis. If isoprenoid biosynthesis was inhibited at an early stage, then the synthesis of colorless carotenoids, the phytol chain of chlorophyll, and the side chain of α-tocopherol should have been inhibited since they originate from a common precursor—i.e., isopentenyl pyrophosphate (Goodwin, 1971). We showed (Table 3–2) that Sandoz 6706 had a very slight effect if any on synthesis of α-tocopherol and phytol of chlorophyll when grown under dim light. Our results indicate that Sandoz 6706 exerted its effect on carotenoid synthesis interfering with dehydrogenation reactions, either by inhibiting catalytic activity of dehydrogenases or by inhibiting formation of a specific dehydrogenase.

CHLOROPLAST DEVELOPMENT: INHIBITION BY SANDOZ 6706

The chlorophyll content of 6706-treated seedlings depended upon the light intensities under which the plants were grown. At 1500 ft-c, the chlorophyll content (Table 3–3) was reduced by 97 percent and these seedlings appeared red, indicating the presence of anthocyanin pigments only. In contrast, treated seedlings grown at 1 ft-c for 6 days appeared a blue-green color and contained about 60 percent as much chlorophyll as did the controls (Table 3–3). When these plants were exposed to 1500 ft-c of light for 12 hours, they lost 80 percent of their original chlorophyll, while control plants accumulated three times more chlorophyll than they originally had. The chlorophyll content of 6706-treated dark-grown plants was determined by the light intensity under which the plants were illuminated. If they were exposed to 1 ft-c of light for 12 hours, the seedlings became green and accumulated about 70 percent as much chlorophyll as the controls (Table 3–3), while those illuminated with 1500 ft-c of light failed to gain chlorophyll pigment.

Table 3-3. Effect of light intensity on chlorophyll and carotenoid content of Sandoz 6706-treated plants[1]

Light regime under which plants were grown	Chlorophyll		Carotenoids	
	Control	Sandoz 6706	Control	Sandoz 6706
	(μg/g fresh weight)			
Light 6 days at 1500 ft-c	790	17	155	1
Light 6 days at 1 ft-c	175	105	45	2
Light 6 days at 1 ft-c, then 12 hours at 1500 ft-c	560	19	40	2
Dark 6 days	—	—	75	3
Dark 6 days, then 12 hours at 1 ft-c	55	41	48	2
Dark 6 days, then 12 hours at 1500 ft-c	62	11	40	1

[1]Seedlings were grown in a 16-hour photoperiod at a temperature of 21°C. High light intensities were provided by fluorescent lamps supplemented with incandescent lamps and low light intensities by incandescent lamps. Concentration of Sandoz 6706 used in these experiments was 0.1 mM.

The carotenoid pigments of the 6706-treated seedlings were virtually absent, irrespective of whether the plants were grown at high or low light intensities or in darkness (Table 3-3). The influence of 6706 on the ribosomal composition of wheat seedlings grown under 1500 ft-c was identical to that of amitrole- and dichlormate-treated seedlings. The ribosomal preparations (Table 3-4) of 6706-treated seedlings had only a single 80 S peak, while control plants contained two peaks with approximate sedimentation coefficients of 70 S and 80 S, which represent chloroplast and cytoplasmic ribosomes, respectively. In contrast, the ribosomal composition (Table 3-4) of 6706-treated seedlings grown for 6 days under 1 ft-c of light showed the presence of both 70 S and 80 S ribosomes; however, the ratio of cytoplasmic 80 S ribosomes to chloroplast 70 S ribosomes for 6706-treated plants was 4:1 as compared with an 80 S to 70 S ratio of 3:1 for controls, thus indicating a slight reduction of 70 S ribosomes. Some of the seedlings grown for 6 days at 1 ft-c were then exposed to 1500 ft-c of light for 12 hours, and a sedimentation analysis showed that the chloroplast ribosomes of these seedlings disappeared while 80 S ribosomes remained (Table 3-4).

In contrast to these results obtained with illuminated plants, both 6706 and control, dark-grown plants were found to have identical ribosomal compositions (Table 3-4), and the ratio of cytoplasmic to chloroplast ribosomes was the same (3:1) in both, indicating that 6706 did not affect accumulation of 70 S ribosomes in the dark. Illumination of these dark-grown plants with 1500 ft-c of light for either 1, 4, or 12 hours caused the rapid reduction and finally the loss of 70 S chloroplast ribosomes present prior to illumination. After 1 hour of illuminating the 6706-treated plants (Table 3-4), their 80 S to 70 S ribo-

Table 3-4. Effect of light intensity of ribosomal content of Sandoz 6706-treated plants[1]

Light regime under which plants were grown	80 S to 70 S ribosome ratios	
	Control	Sandoz 6706
Light 6 days at 1500 ft-c	2:1	80 only
Light 6 days at 1 ft-c	3:1	4:1
Light 6 days at 1 ft-c, then 12 hours at 1500 ft-c	3:1	80 only
Dark 6 days	3:1	3:1
Dark 6 days, then 1500 ft-c for 1 hour	3:1	4:1
Dark 6 days, then 1500 ft-c for 4 hours	2:1	6:1
Dark 6 days, then 1500 ft-c for 12 hours	2:1	80 only
Dark 6 days, then 12 hours at 1 ft-c	2:1	2:1
Isolated ribosomes incubated with Sandoz 6706 for 6 hours, 1500 ft-c	2:1	2:1

[1]Plants grown as described in Table 3-3.

somal ratio changed to 4:1, as compared with a 3:1 ratio for dark-grown 6706-treated plants. Four hours of illumination of 6706-treated plants changed the 80 S to 70 S ratio to 6:1, and after 12 hours of light only a hint of 70 S ribosomes was observed (Table 3-4) in the 6706 plants. The 80 S to 70 S ratio (2:1) for control seedlings was about the same for each light treatment.

In contrast, when dark-grown plants were illuminated with low intensities of light (1 ft-c) for up to 12 hours, the 70 S ribosomes were not reduced, and the 80 S to 70 S ratio (2:1) was the same as that of the controls (Table 3-4). However, when the light intensity was again increased (75 ft-c), the 80 S to 70 S ratio dropped to 6:1.

These results suggest that 6706 in conjunction with light causes rapid destruction of 70 S chloroplast ribosomes. To determine if the destruction of 70 S ribosomes was caused by the photoactivation of the herbicide, isolated 70 S and 80 S ribosomes were incubated with 6706 *in vitro* under 1500 ft-c of light for 6 hours. The 70 S ribosomes were not destroyed, and 80 S to 70 S ratios were identical in both the control and treated extracts (Table 3-4). The ultrastructural effect of 6706 on light-grown (1500 ft-c) and dark-grown seedlings appeared to be identical with that of aminotriazole and dichlormate. Plastids of light-grown plants lacked grana (G) and ribosomes, but contained thylakoids that were unbranched, unusually long, and arranged parallel to the edge of the plastid envelope. The etioplasts of 6706 dark-grown plants were morphologically identical with the etioplasts of control plants, each having prolamellar bodies (P) and ribosomes.

When 6-day-old dark-grown plants were exposed to high light intensity (1500 ft-c) for 12 hours, the control seedlings became green, whereas the 6706-

treated plants remained white. The P of the etioplast from this white tissue changed during the greening process into an aggregated unit of highly disorganized, interconnecting membranes rather than the normal structure of G-thylakoids, and the stroma became devoid of ribosomes. The plastid appeared to be morphologically similar to dichlormate-treated plants grown under the same conditions.

When seedlings were grown under 1 ft-c of light for 6 days, the plastids (Figure 3–2) of control plants contained P, ribosomes, and G structures, while plastids of 6706-treated plants also had P and ribosomes but lacked G structures (Figure 3–3).

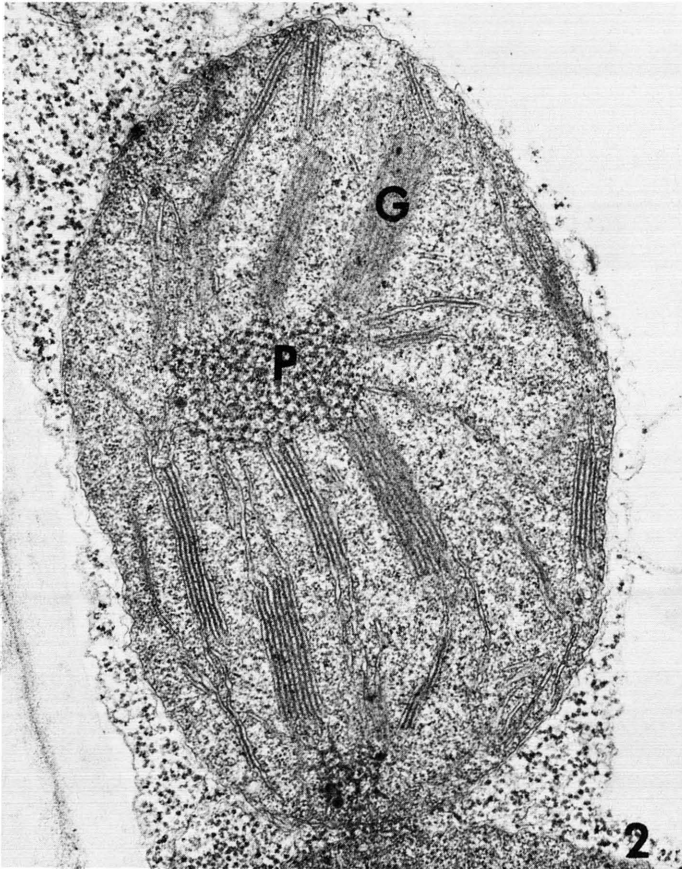

Figure 3–2. Chloroplast from control plant grown under 1 ft-c of light for 6 days. Note grana (G) structures surrounding the prolamellar body (P). × 31,000.

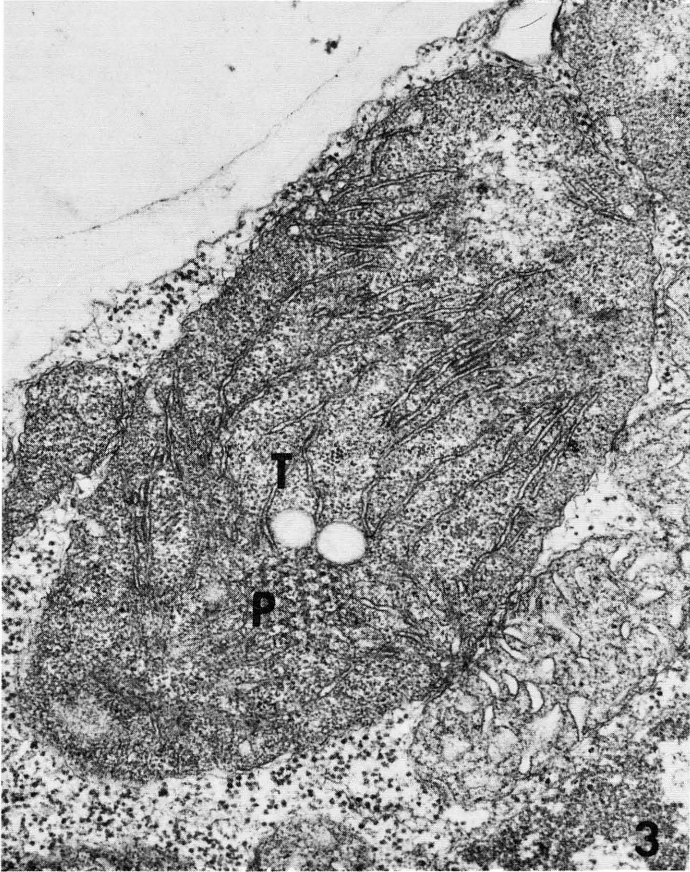

Figure 3-3. Chloroplast from Sandoz 6706-treated plant grown under 1 ft-c for 6 days. Note absence of grana. Note single thylakoids (T) radiating from prolamellar body (P). × 22,000.

We infer that chlorophyll pigments in the absence of carotenoids become susceptible to photo-oxidation. These pigments may be converted to highly reactive molecular species, which then interact with and destroy other chloroplast components. Our data suggest that unstabilized photosensitized chlorophyll or precursors of chlorophyll react directly or indirectly with 70 S ribosomes and thylakoids to oxidize and destroy them. Some evidence for this inference is supported by the work of Leff and Krinsky (1967), who report that photo-oxidized chlorophyll could function as a photosensitizer that reacts with chloroplast DNA and results in genetic alteration.

SUMMARY

Sandoz 6706 inhibited the synthesis of both carotenes and xanthophylls and caused a massive accumulation of phytoene in wheat seedlings. The synthesis of chlorophyll and tocopherol was not inhibited in wheat, even though these substances have many biosynthetic reactions in common with the carotenoids. Our results indicate that 6706 interfered with the dehydrogenation reactions of the carotenoid pathway. Chloroplast ribosomes and thylakoids were absent in the light-grown treated seedlings. Unstabilized photosensitized chlorophyll may react with these chloroplast structures and destroy them.

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Chapter 4. Growth-Mediated Response of Methyleneoxindole with a Proposed Mechanism of Action for 2,4-D

V. A. McMahon and C. L. Villemez

INTRODUCTION

The present study was undertaken to determine (a) how auxin promotes cell elongation and (b) if indoleacetic acid (IAA) or its oxidation products are directly responsible for growth-promoting activity. In view of the types of responses that 2,4-dichlorophenoxyacetic acid (2,4-D) and IAA have in common, it can be suggested that 2,4-D might elicit its response through some phase of IAA metabolism (Zimmerman, 1951; Lee, 1972).

LITERATURE REVIEW

Direct Effect of 2,4-D on an Enzyme

Van Der Woude et al. (1972) demonstrated a 10- to 30-percent increase in β-1,3-glucan synthetase activity in the presence of 5 μM 2,4-D *in vitro*. This enzyme is reported to have been isolated in plasma membrane from onion stems. These authors used a differential staining technique that characteristically stains the plant plasma membrane to identify cell fraction purity. In their paper, Van Der Woude et al. mention a personal communication of C. L. Villemez, discussing stimulation of cellulose synthesis in the presence of IAA *in vitro*. This β-1,4-glucan synthetase activity was also found in the particulate fraction from mung bean seedlings.

IAA Oxidizing Enzymes and Effect of 2,4-D on Methyleneoxindole Reductase

An oxidation product of IAA—3-methyleneoxindole (MeOx)—is reportedly 10 to 1000 times more effective in stimulating growth of higher plants than IAA (Tuli and Moyed, 1969; Basu and Tuli, 1972*a*; McMahon, unpublished results). Furthermore, the subsequent reduction of MeOx to methyloxindole is

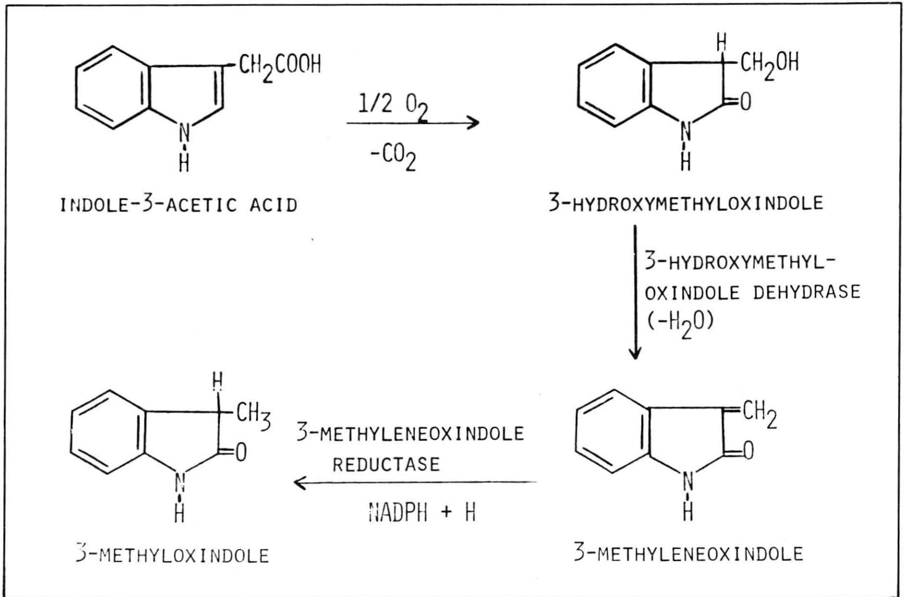

Figure 4-1. Oxindole pathway of IAA metabolism in plants (scheme of Basu and Tuli, 1972*b*).

catalyzed by at least two NADPH-linked methyleneoxindole reductases (Moyed and Williamson, 1967). Methyloxindole is an inert metabolite of MeOx. One of these reductases, reductase A, is noncompetitively inhibited by 2,4-D, while 2,4-D is a weak competitive inhibitor of reductase B.

Evidence for the existence of an oxindole pathway of IAA metabolism in higher plants is found by the presence of an IAA oxidation product of MeOx in a cell-free system (Hager and Schmidt, 1968) and by the isolation of the enzymes associated with this pathway. The proposed scheme shown in Figure 4-1 is from Basu and Tuli (1972*b*), who have isolated a partially purified 3-hydroxymethyloxindole dehydrase that catalyzes the dehydration of 3-hydroxymethyloxindole (HMO) to MeOx. Previously, workers in the same laboratory were able to demonstrate the oxidation of IAA to HMO and MeOx using cell-free extract from pea seedlings (Still et al., 1965; Tuli and Moyed, 1967).

GROWTH OF *Phaseolus aureus* ON METHYLENEOXINDOLE

Growth-Promoting Activity

Several problems were encountered in using mung bean stem segments to test for promotion of cell elongation. The problems were ameliorated by: (a)

carefully selecting seedlings 3 cm tall, since maximum elongation rates of seedlings occur between 3 and 6 cm; (b) using dim light in harvesting segments of mung bean; (c) preincubating segments in 0.02 M potassium phosphate buffer to reduce endogenous auxin levels; and (d) using stock solutions of MeOx at not greater than 1×10^{-4} M concentration, because MeOx is reported to polymerize above concentrations of 1×10^{-3} M (Hinman and Bauman, 1964). These precautionary measures have allowed demonstration of growth in 7-mm stem segments excised from the growing tip of etiolated mung bean seedlings (Figure 4-2).

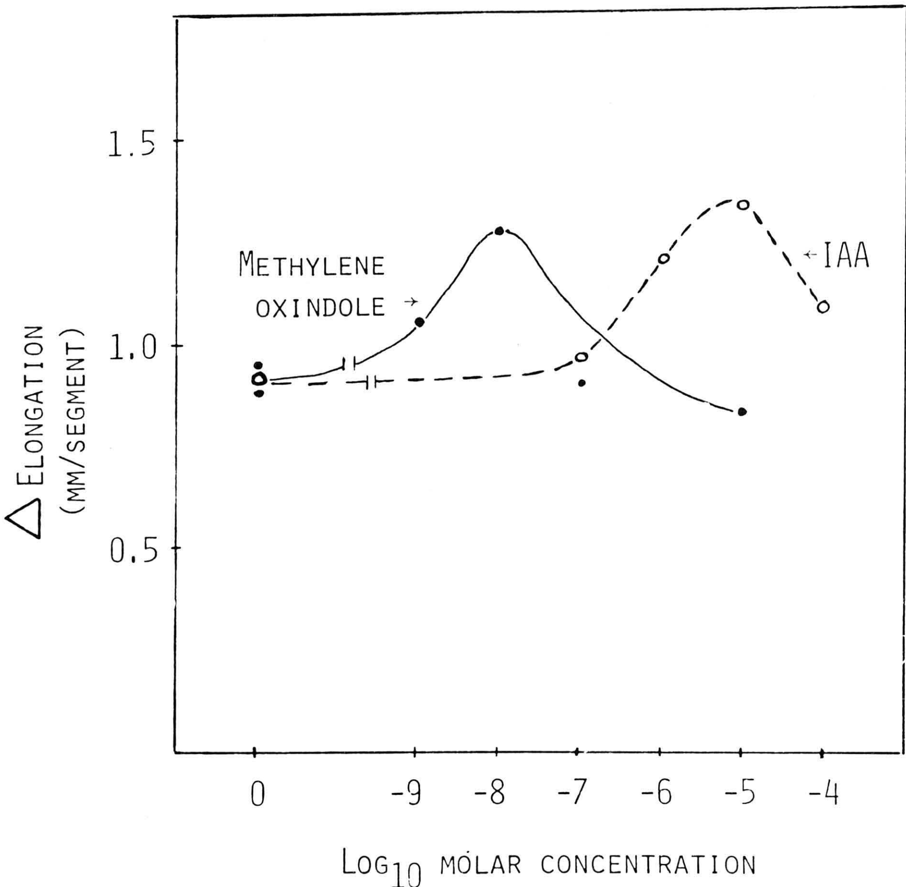

Figure 4-2. Effect of MeOx and IAA on elongation of etiolated mung bean stem segments (7 mm). Segments were cut in dim light, preincubated 4 hours in 0.02 M phosphate buffer (pH 6.5), and exposed to auxin with the same concentration of buffer for 19 hours; measurements of change in elongation were made with a calibrated magnifying lens. Each point represents an average value for 10 segments.

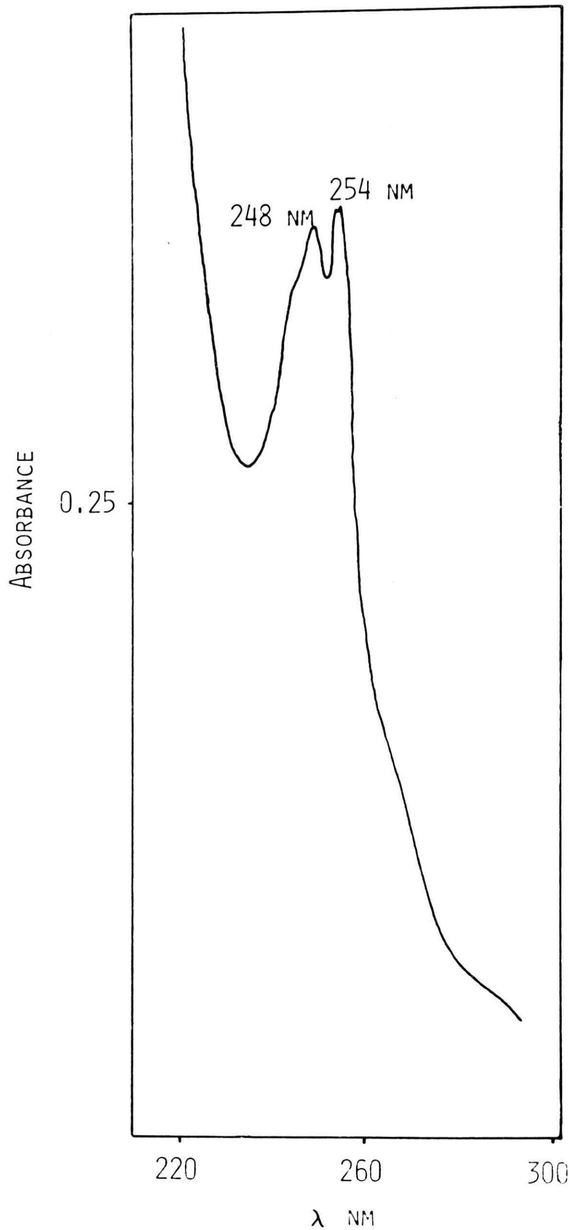

Figure 4–3. UV absorption spectrum of MeOx in water; concentration of 1.3×10^{-5} M. This compound was separated by paper chromatography in a solvent of 95:5, v/v water/isopropanol (Rf = 0.47).

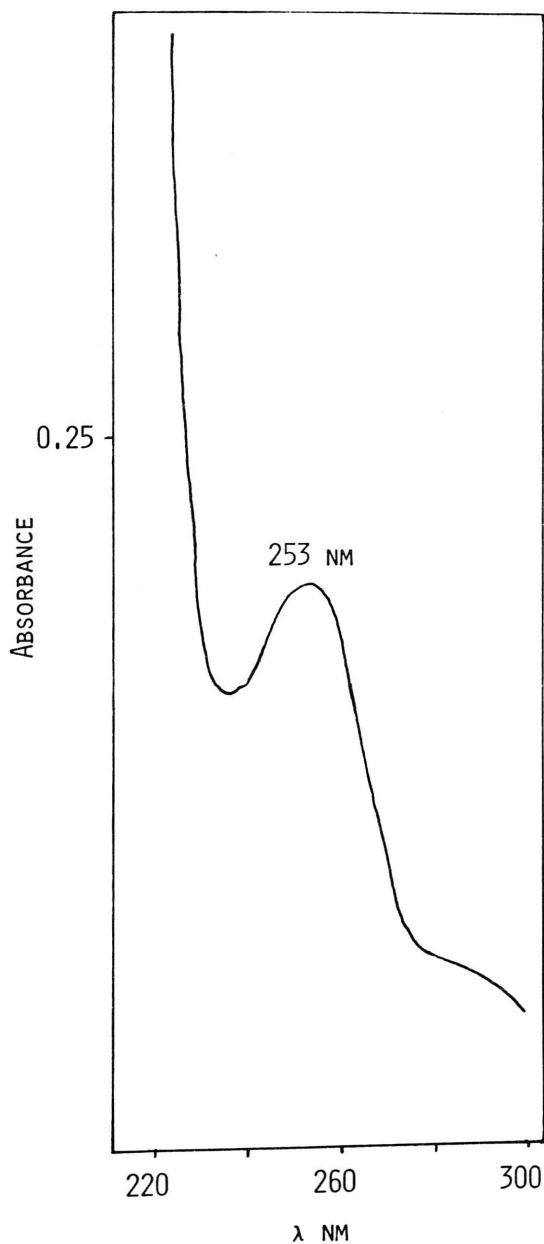

Figure 4–4. UV absorption spectrum of MeOx in water; concentration of 2.4×10^{-5} M. This compound was separated by paper chromatography in a solvent of 95:5, v/v water/isopropanol (Rf = 0.86).

Apical segments of mung bean stems (7 mm) were incubated in either IAA or MeOx at various concentrations. Figure 4–2 shows that the optimum concentration of IAA for growth was 10 to 100 μM, while MeOx showed optimum concentration at 0.1 to 0.01 μM—a thousandfold difference in concentration. These growth studies were best mediated by mung bean seedlings 3 to 5 cm tall. Results suggest that MeOx may be the substance that promotes growth in mung bean stems.

Synthesis of Methyleneoxindole

MeOx was prepared after the procedure of Hinman and Bauman (1964). IAA was reacted with *N*-bromosuccinimide to yield a recrystallized product of 3-bromooxindole-3-acetic acid. This product is converted to MeOx (Figure 4–3) in the presence of water (this is readily accomplished by paper chromatography in 95:5, v/v water/isopropanol). The reaction occurs simultaneously, and unwanted products of 3-methyloxindole (Figure 4–4, Rf 0.86) and possibly a polymeric form of MeOx (Rf = 0.08 in 95:5, v/v water/isopropanol, paper chromatography) are separated from MeOx. MeOx is readily visualized under ultraviolet (UV) light and subsequently dissolved in dilute solution in distilled water.

A POSSIBLE MECHANISM OF ACTION FOR 2,4-D

It is uncertain whether 2,4-D in low concentrations exerts its effects on cell elongation directly, or whether 2,4-D might change the level of endogenous auxin. Since MeOx, rather than IAA, is likely to be the natural growth-mediator hormone, the endogenous level of MeOx possibly is the auxin of reference. To test this hypothesis, chlorogenic acid (a growth inhibitor that also blocks oxidation of IAA to MeOx) could be employed to inhibit the conversion of IAA to MeOx in stem segments of mung bean, and sufficiently aged, treated segments should lack MeOx-mediated growth. Combinations of 2,4-D with MeOx and appropriate controls should provide evidence of direct or indirect effects of 2,4-D on growth; if the 2,4-D effect is indirect, MeOx should have a longer growth-promoting effect than its control (assuming that MeOx reductases are inhibited by 2,4-D).

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Chapter 5. Cytological and Biochemical Effects of Trifluralin on Mitosis

P. G. Bartels, F. D. Hess, and D. E. Bayer

INTRODUCTION

The objectives of this study were: (a) to investigate the ultrastructural effects of trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine) on cell division in wheat, corn, and cotton root tips; (b) to investigate the influence of trifluralin on the *in vitro* polymerization of microtubules from pig-brain protein subunits; (c) to determine if radioactively labeled trifluralin binds to purified pig-brain microtubules; and (d) to compare the mode of action of trifluralin with that of colchicine in root-tip cells.

Trifluralin is widely used as a soil-active preemergence herbicide for controlling annual weeds in cotton, soybean, safflower, and many vegetable crops. Best results are normally obtained when it is incorporated in soil to a depth of 2 to 4 inches. Movement of the herbicide within the plant is limited, and it appears to be retained in the roots. No evidence was found of an active accumulation of ^{14}C-trifluralin by cotton and soybean roots; rather, accumulation was attributed to adsorption in cuticle, epidermis, and cell walls (Strang and Rogers, 1971). Growth of the entire plant is affected by trifluralin, however, apparently by interference with root growth and development. Trifluralin has been reported to interfere with root development in a number of plant species (Ashton and Crafts, 1973). Roots of treated plants appear swollen, stunted, and malformed. After 12 hours of trifluralin treatment, abnormal cell enlargement (enhanced radial expansion) occurs in cells of the cortex (Mallory and Bayer, 1972). This enlargement in the region of cell elongation is thought to produce the commonly observed club-shaped root tips. Enhanced radial expansion compensates for the inhibited elongation, resulting in approximately the same volume change for treated and untreated tissue (Bayer et al., 1967).

Lower application rates of trifluralin to roots appear to have no marked effect on the apical meristem of the root but strongly inhibit formation of lateral roots. Trifluralin induces an expansion of the pericycle tissue, particularly in the region opposite the protoxylem, and, at the same time, restricts the number of layers of pericycle cells. The net result is the inhibited formation of lateral root primordia, resulting from suppressed division or activity of pericycle cells

(Bayer et al., 1967; Hacskaylo and Amato, 1968). Higher rates of trifluralin inhibit both apical meristems and lateral roots.

LITERATURE REVIEW

Observations of stunting, swelling of root tips, and inhibition of lateral root formation in plants treated with trifluralin suggest marked cytological malfunction. Cytological studies of trifluralin-treated roots showed that mitosis or cell division was affected or disrupted. In addition to these mitotic aberrancies, differentiation of the vascular cylinder is reported to occur abnormally near the distal portion of the primary roots (Mallory and Bayer, 1972). The type of mitotic figure prevailing in trifluralin-treated tissue seems to depend on plant species, concentration of herbicide reaching the cells (or uneven distribution of herbicide among the cells of the tissue), and time of herbicide application to the plant. In soybean root, Talbert (1965) observed an increase in cells in prophase and evidence of only a few other mitotic stages. Lignowski and Scott (1972) stated that prophase did not appear to be affected by trifluralin; they found prophase appeared normal after 12 hours of treatment, but they also observed an increase in cells in metaphase. Polyploid and multinucleate cells were observed after these tissues had been treated for 24 hours. Bayer et al. (1967) found that no single stage of mitosis was predominant in trifluralin-treated onion root-tip cells. Mitotic activity was not disrupted to the same extent in all cells, and some appeared to undergo normal mitosis. Jackson and Stetler (1973) observed an increase in prophase of treated endosperm cells of African blood lilies, and their results corresponded with the findings of Talbert (1965). They suggested that destruction of microtubules during prophase may be responsible for accumulation of cells in prophase.

Typical mitotic aberrations found in trifluralin-treated root cells were c-pairs, enlarged amoeboid nuclei, micronuclei, and polyploid plants. These aberrations were similar to those observed with colchicine, a mitotic poison that has been used for crop improvement through development of polyploid plants (Eigsti and Dustin, 1955). Colchicine modifies microtubules in plant and animal cells, and is thought to prevent formation of microtubules by stoichiometrically binding to a site of interaction through which the protein subunits combine to form microtubules (Adelman et al., 1968). It has been reported, however, that the concentration of colchicine required to block cell division in plant cells is 1000 times higher than the concentration required to block cell division in animal cells (Hepler and Palevitz, 1974).

In this study, cell division of trifluralin-treated wheat, corn, and cotton root-tip cells was examined at the ultrastructural and biochemical levels to determine the mode of action of the herbicide on microtubules of root cells.

ULTRASTRUCTURAL ANALYSIS OF TRIFLURALIN-TREATED AND CONTROL ROOT-TIP CELLS

Cytological studies of trifluralin-treated roots showed that mitosis was inhibited and that typical mitotic aberrations of c-pairs and enlarged amoeboid nuclei followed. The type of mitotic figure prevailing in trifluralin-treated roots seems to depend on plant species and concentration of herbicide used. In a detailed ultrastructural study of the effect of colchicine on mitosis of cells in wheat root tips, Pickett-Heaps (1967) stated, "Microtubules disappeared from spindle no matter what stage in mitotic cycle had been reached prior to colchicine application." The electron microscope methods used in our ultrastructural research to study microtubule-trifluralin interactions were described by Bartels and Hilton (1973) and Hess and Bayer (1974).

Microtubules of Corn, Wheat, and Cotton Root Tips

Control of untreated root cells. Ultrastructural analysis of root tips revealed that cortical and spindle microtubules were readily observed. During nuclear division of mitosis, microtubules were observed near the condensed chromatin by late prophase; at metaphase, they were attached to chromosome kinetochores (Figure 5-1), and extended from pole to pole without chromosome attachment. During anaphase and telophase, spindle microtubules were observed in the interzone between the two sets of daughter chromosomes. They

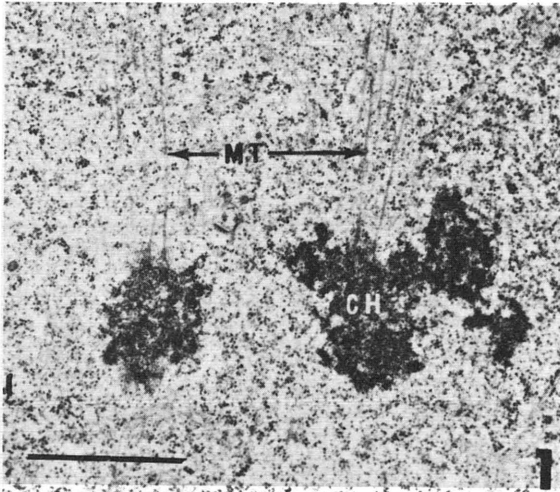

Figure 5-1. Metaphase chromosomes arranged along the equatorial plate with microtubules attached to kinetochores. Microtubules radiate toward poles of the spindle apparatus. Scale marker equals 1.0 μm × 21,000.

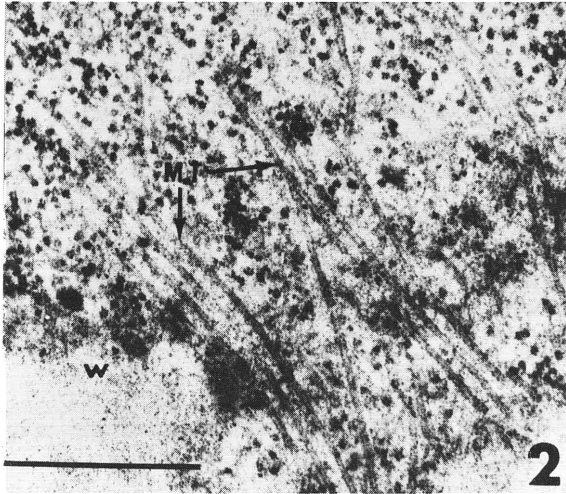

Figure 5–2. Microtubules adjacent to a longitudinal cell wall in an interphase cell. Scale marker equals 0.5 μm × 53,400.

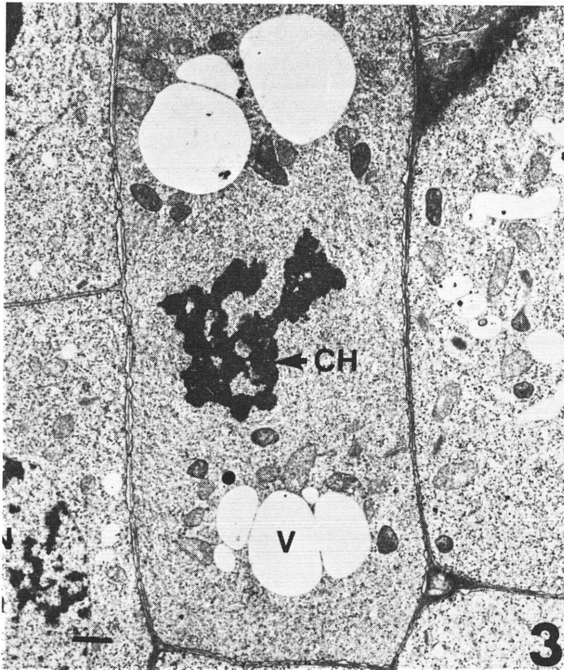

Figure 5–3. Arrested metaphase division figures resulting from microtubular disappearance at late prophase or early metaphase. Scale marker equals 1.0 μm × 5000.

were oriented roughly parallel to one another and were normal to the plane of cell plate. The cell plate consisted of many vesicles that appeared to be fusing.

In the interphase cell, microtubules were abundant near cell walls (Figure 5–2), lying immediately beneath the primary wall with their orientation circumferential to the cell axis. These structures, cortical microtubules, may control microfibril orientation in the cell wall. There was no evidence of any ultrastructural difference between spindle and cortical microtubules; therefore, they will be considered different only in location in the cell. A detailed report on the role of microtubules has been published by Newcomb (1969).

Trifluralin-treated cells: microtubules absent. At any one time, cells at the tips of roots were observed to be in different stages of mitosis; therefore, the initial disappearance of the microtubules would affect different stages of cell division. Mitosis stopped when the microtubules disappeared. With the mitotic sequence unable to proceed, nuclear envelope reformation occurred in some cells and yielded interphase nuclear patterns characteristic of the stage at which mitosis was arrested. Nuclear divisions stopped at late prophase or early metaphase were characterized by chromosomes arranged in an abnormally

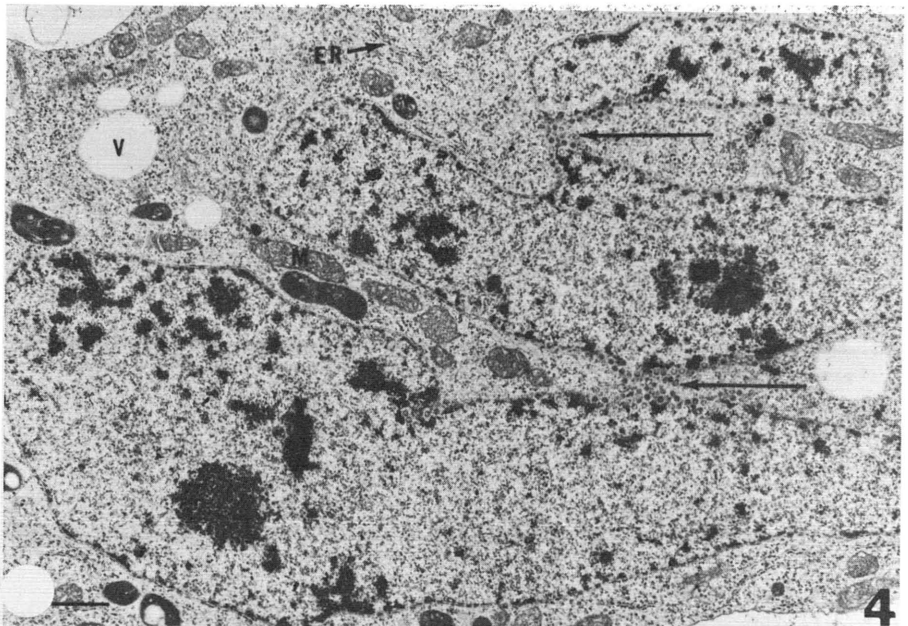

Figure 5–4. Nucleus after nuclear reformation of a blocked metaphase. Note thin connections (arrows) between each of the nuclear segments, indicating that this is a uninucleate and not a trinucleate. Scale marker equals 1.0 μm × 8500.

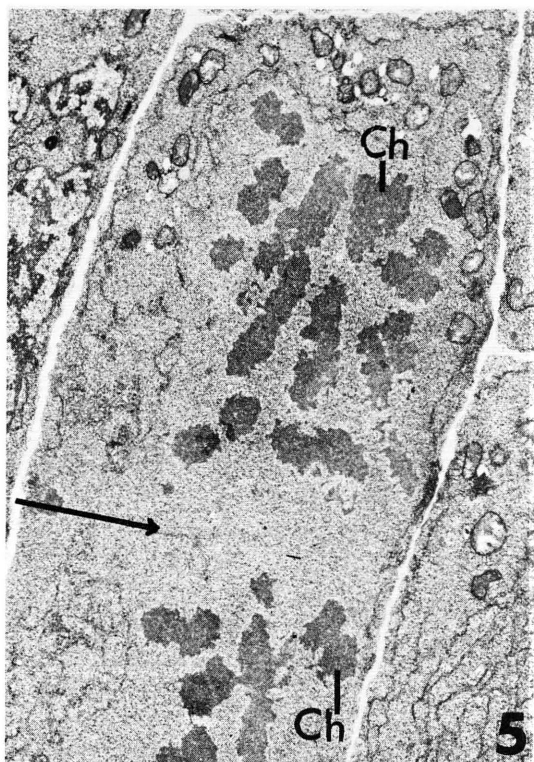

Figure 5–5. Wheat root cell in telophase treated with trifluralin for 3 hours showed no evidence of cell plate or microtubules. A few cell membranes can be seen (arrow). Daughter chromosomes (Ch) are located at each end of the root cell. × 6000.

small group in the clear zone of cotton root cells (Figure 5–3), or by chromosomes spread throughout the central region of the cytoplasm in wheat roots. Spindle microtubules were not observed in cotton, wheat, or corn root meristem cells. If nuclear envelope re-formation occurred, polyploid nuclei resulted. Many of the re-formed nuclei were highly lobed (Figure 5–4). Chromosomes in cells arrested at late anaphase or early telophase remained in two distinct groups located at each end of the root cell (Figure 5–5). Both cell plate vesicles and spindle microtubules were absent in the interzone between daughter chromosomes. Without vesicles, cell plate formation did not occur, and this resulted in binucleate cells. Incomplete or partially formed cell cross-walls were occasionally found between the daughter chromosomes (Figure 5–6), resulting from partial fusion of some cell plate vesicles.

Microtubule absence in interphase cells did not stop occurrence of mitotic division attempts. The mitotic cycle appeared normal until microtubules were

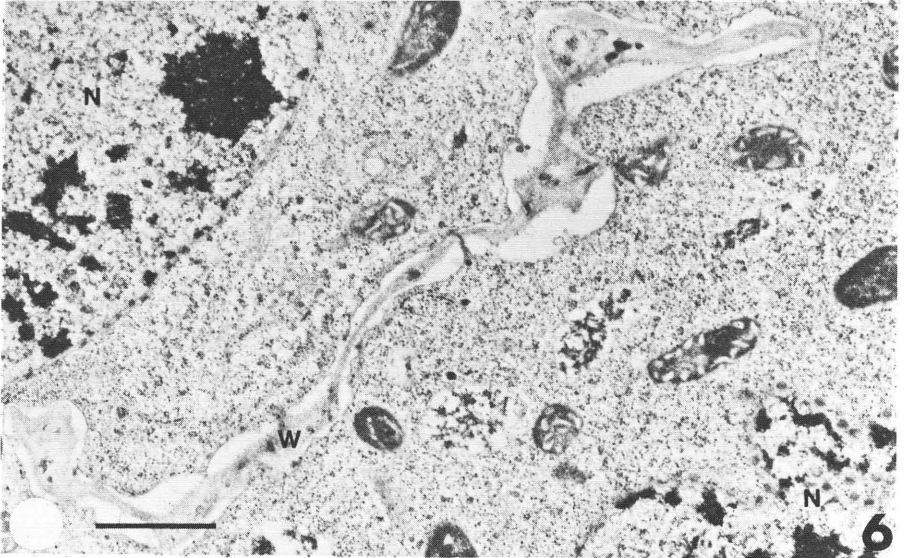

Figure 5-6. Disruption of cell division at telophase due to disappearance of microtubules. The cell wall has a distorted appearance and extension has ceased at both ends of the wall. Scale marker equals 1.0 μm × 18,000.

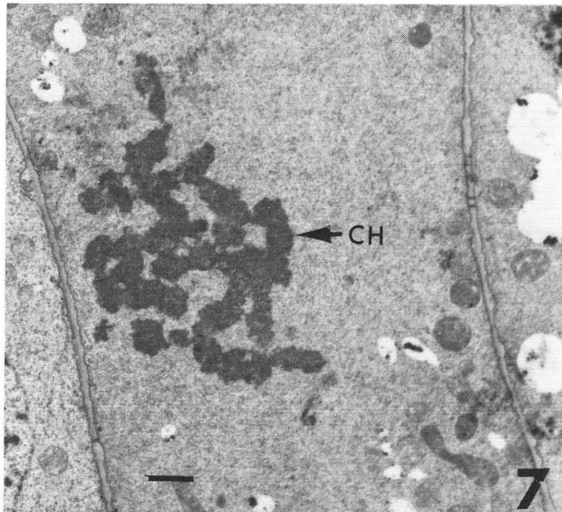

Figure 5-7. Arrested metaphase division figures in cells containing no microtubules, with the division sequence terminated in an aggregated chromosome configuration. Scale marker equals 1.0 μm × 5800.

involved. The chromosomes condensed and divided in a normal manner, and the nuclear envelope began to disperse as prophase progressed. By late prophase, no microtubules were present near the condensed chromatin, although they had been observed at this stage in untreated tissue. The chromosomes did not align along the equatorial plate at metaphase but instead coalesced in the clear zone (Figure 5–7), causing the division sequence to be arrested. Occasionally, one or more chromosomes became separated from the main group. Nuclear envelope reformation around arrested division figures resulted in polyploid polymorphic nuclei (Figure 5–4).

Trifluralin-treated cells: microtubules present. Microtubules were present in some root cells but in fewer numbers than in untreated cells at the same stage in the cell cycle. A few cortical microtubules were observed following a 3-hour trifluralin treatment, but they disappeared after 12 or more hours in wheat or corn seedlings. In cotton, some microtubules were observed in some root meristem areas, even after herbicide treatment for 96 hours. If microtubules were present along the wall in interphase cells, they also occurred in the nuclear divisions of adjacent cells. In some instances, microtubules present during cell division were abnormally oriented (Figure 5–8). During metaphase, disoriented microtubules were always found near the chromosomes, with some exhibiting kinetochore attachments (Figure 5–8).

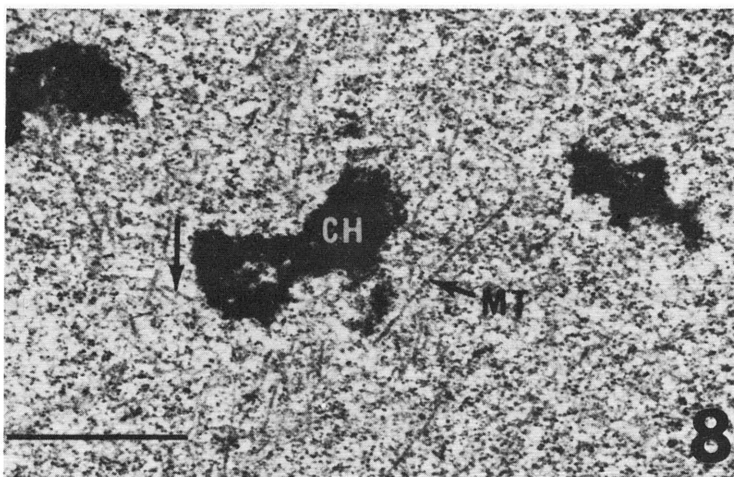

Figure 5–8. Trifluralin treatment has not caused complete disappearance of microtubules in this metaphase division figure. Chromosomes are aligned along a metaphase plate, but microtubules seem to be radiating in all directions from chromosomes. Short microtubules (arrow) are a result of their oblique orientation with respect to plane of sectioning (or they may be genuinely shortened as a result of contraction of breakdown). Scale marker equals 1.0 μm × 18,700.

Other Cellular Structures in Treated Roots

Other organelles of the root cells in cotton plants appeared not to be morphologically affected by trifluralin but were altered in wheat and corn roots. Within 12 hours, abnormally large vacuoles developed in the root-tip cells (Figure 5-9); these cells enlarged radially rather than longitudinally. Also, some of the endoplasmic reticulum (ER) cisternae became swollen and appeared to accumulate next to the cell wall (Figure 5-10). Other cytoplasmic organelles, such as ribosomes, plastids, and mitochondria, did not appear to be structurally modified by the herbicide.

IN VITRO INTERACTION BETWEEN ISOLATED MICROTUBULES AND TRIFLURALIN

Loss of microtubules from trifluralin-treated root cells may have resulted from a direct action of the herbicide on polymerization of microtubules, as

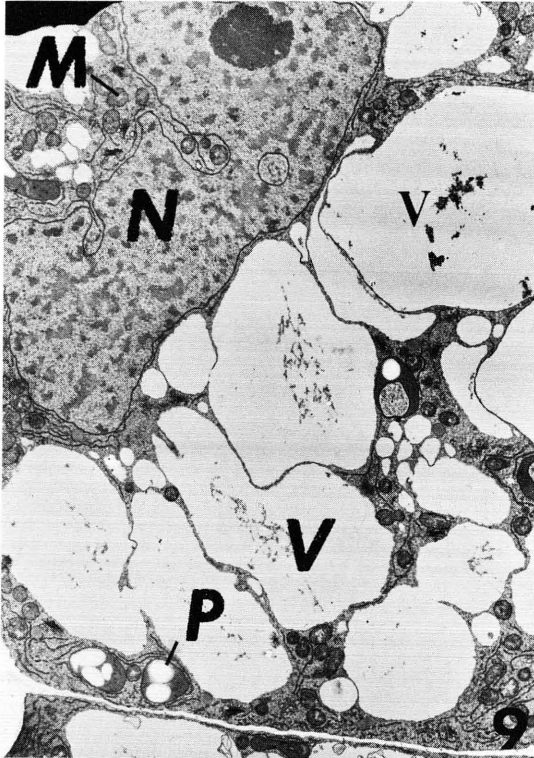

Figure 5-9. Cell from wheat root with highly lobed nucleus (N) and large vacuoles (V). ×3700.

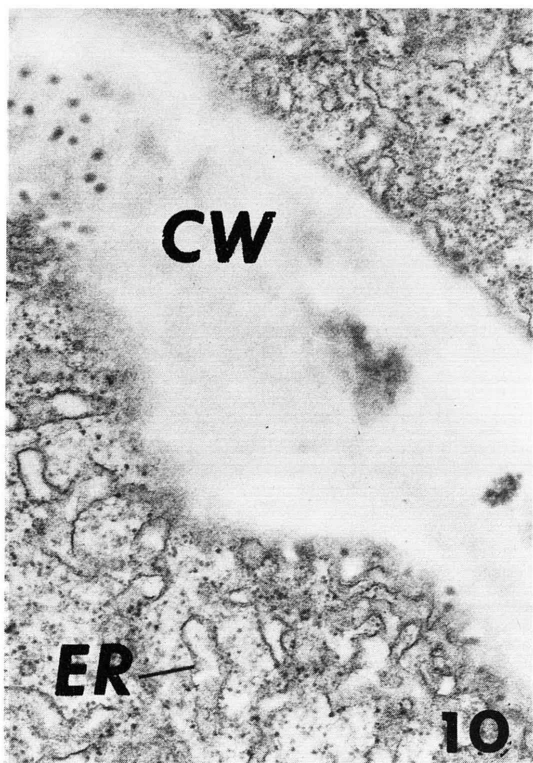

Figure 5-10. Part of a corn root cell treated for 72 hours. Endoplasmic reticulum (ER) cisternae located next to cell wall (CW) was swollen. × 39,000.

has been found for colchicine. To test this hypothesis, Bartels and Hilton (1973) attempted to isolate and purify microtubules from corn root tips in order to use these tubulin in polymerization experiments with trifluralin or colchicine. Results, however, were unsatisfactory. Colchicine binding was used as a sensitive measure of the amount of tubulin in the plant extracts, but very little colchicine binding to plant protein was observed. Hart and Sabnis (1973) also reported that they could not isolate or detect any colchicine-binding activity in nonvascular tissue of higher plants. The addition of higher plant extracts to sheep-brain *(Ovis musimon)* microtubular preparations did not interfere with binding of radioactively labeled colchicine to brain microtubules. They concluded that microtubules of higher plants were probably present only in small quantities and were much less stable *in vitro*. Therefore, Bartels and Hilton (1973) used pig-brain tissue as a source of microtubular protein.

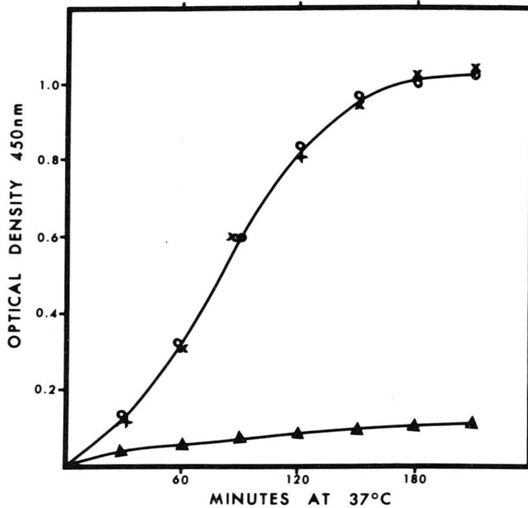

Figure 5-11. Effect of trifluralin and colchicine on aggregation of microtubule subunits. Incubation medium: 3 mg protein/ml; 37°C; KPO_4-KCl-GTP buffer. Legend: (o–o) control; (×–×) saturated concentration of trifluralin; (Δ–Δ) colchicine.

Bartels and Hilton assumed that microtubules of brain tissue and root tips were similar, and that results obtained with brain microtubules were applicable to root tissue. Both animal and plant microtubules disappear in the presence of colchicine and at 0°C (Pickett-Heaps, 1967; Borisy and Olmsted, 1972). Structurally, animal and plant microtubules are similar—both are hollow cylinders about 250 Å in diameter, running a straight course through the cytoplasm. All tubulin appear to be similar in their molecular weight, sedimentation coefficient, and guanosine triphosphate binding.

Purified tubulin from pig-brain tissue (Borisy et al., 1972) was polymerized in the absence or presence of trifluralin or colchicine. The curve in Figure 5-11 indicates, by open circles, aggregation in the absence of herbicides or colchicine; this curve has a characteristic sigmoid shape. Colchicine (100 μM) suppressed the development of turbidity (Figure 5-11) by inhibiting the association of the microtubular subunits. In contrast, a saturated solution of trifluralin did not prevent aggregation of subunits (Figure 5-11). These curves were identical to the control curves.

Because these linear aggregates were not morphologically similar to *in vivo* microtubules, a second method of *in vitro* polymerization was used (Borisy and Olmsted, 1972). When viewed with the electron microscope, the products of this polymerization were found to be microtubular. Figure 5-12 is a representative picture of microtubules formed *in vitro* in the absence or presence of

saturating concentrations of trifluralin; it shows that these herbicides do not inhibit animal microtubular assembly. Colchicine (100 μ M) and cold temperature inhibited the formation of microtubules (Borisy and Olmsted, 1972).

To determine whether herbicides bind to animal tubulin, purified tubulin from pig-brain tissue was incubated at 37°C with radioactively labeled ^{14}C-trifluralin, ^{14}C-chlorpropham (isopropyl *m*-chlorocarbanilate), and ^{14}C-pronamide [3,5-dichloro(*N*-1,1-dimethyl-2-propynyl)benzamide]. Assay of binding activity was measured by the gel filtration method. Binding of radioactively labeled ^{14}C-colchicine to tubulin was taken to represent a maximum of 100 percent binding. Using this criterion, binding of the ^{14}C-chlorpropham, trifluralin, and pronamide was 4.4, 3.5, and 2 percent, respectively (Table 5–1). Weisenberg et al. (1968) reported that 1 molecule of colchicine binds with 1

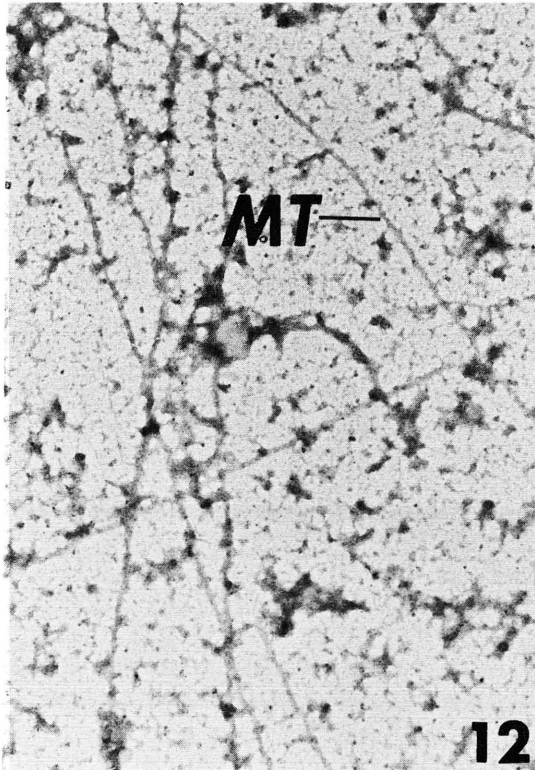

Figure 5–12. Low magnification view of a field of repolymerized pig-brain microtubules (MT). Polymerization medium incubated 20 min at 37°C contained saturated concentration of trifluralin plus PIPES-EGTA-Mg SO_4-GTP buffer. × 21,800.

Table 5-1. Herbicide- and colchicine-binding activity to purified microtubular protein assayed by gel filtration method[1]

Herbicide and specific activity	Cpm bound per mg protein	Specific activity herbicide/ specific activity colchicine (%)
[^{14}C]-pronamide (1.4 μCi/mg)	480	2.0
[^{14}C]-trifluralin (40 μCi/mg)	850	3.5
[^{14}C]-chlorpropham (20 μCi/mg)	970	4.4
[^{14}C]-colchicine (38 μCi/mg)	24,500	100

[1]Incubation medium: 2 mg protein in 1 ml KPO_4-KCl buffer pH 6.8, GTP, Mg^{+2}, 1 μCi of [^{14}C]-herbicide, or [^{14}C]-colchicine; 37°C for 1 hour.

microtubular dimer. Results indicated very little interaction between the herbicides and animal tubulin, which may explain why the herbicides did not interfere with polymerization of the microtubules. Saturating herbicidal concentrations interfered with neither polymerization of microtubules nor binding of ^{3}H-colchicine (1μCi) to the microtubular proteins. ^{3}H-colchicine bound 82,500 cpm/mg protein. Trifluralin or pronamide plus ^{3}H-colchicine bound 82,500 cpm/mg protein (the same amount). In this study, the amount of colchicine binding was assayed by the DEAE cellulose filter method.

Even though very little binding of radioactively labeled herbicides to purified tubulin from pig-brain tissue was observed, ^{14}C-trifluralin did bind to protein in the crude 100,000 g supernatant from corn root tips (4300 cpm/mg). The type or nature of the protein that bound the trifluralin is unknown. No evidence was found that the binding protein consisted of sulfhydryl protein of roots, as suggested by the work of Shahied and Giddens (1970). The herbicide may have bound to a lipid component in the supernatant.

MODE OF ACTION OF TRIFLURALIN ON MITOSIS: THEORIES

Observations indicate that trifluralin treatment often induces a range of effects from near-normal mitosis to severe colchicine-like, disrupted mitosis. The severity of trifluralin's effect on microtubules of root meristem cells probably depends on the herbicide concentration reaching each cell and on the herbicide's mobility. Hess and Bayer (1974) proposed that the concentration of

trifluralin required for complete microtubular disappearance—and thus complete blockage of mitosis—is near an aqueous saturated concentration. Therefore, the degree of saturation achieved would determine the thoroughness of microtubular disappearance and, hence, the degree of mitotic disruption. In some roots, trifluralin accumulates in oil duct gland cells, thus effectively blocking its movement to these dividing cells (Mallory and Bayer, 1972).

The ultrastructural effects of trifluralin and of colchicine on root cells appear to be similar. Light and electron microscope studies revealed that trifluralin produced both chromosomal and mitotic aberrations similar to those caused by colchicine (which prevents formation of microtubules). Not all herbicides that produce mitotic disruptions cause loss of microtubules. Hepler and Jackson (1969) reported that propham at 10 ppmw did not destroy microtubules as did colchicine, but rather led to their disorientation in the cell. Ultrastructural studies showed that trifluralin at aqueous saturated concentrations caused disappearance of microtubules in a manner similar to colchicine. To determine if trifluralin acted like colchicine at the biochemical level, the herbicide was incubated with two *in vitro* microtubular-forming systems from pig-brain tissue. Results showed that trifluralin failed to inhibit the aggregation and polymerization of animal microtubules. The binding experiments, with radioactively labeled herbicides and colchicine, showed that the herbicides did not bind to animal tubulin, nor did they denature the colchicine-binding site on microtubules. Biochemical results showed that trifluralin did not disrupt the microtubules *per se*, nor did it exert its effect by direct interaction with animal microtubular protein. To date, tubulin has not been isolated and purified from a higher plant source; thus, the interaction of trifluralin with higher plant tubulin has not been studied. Current research on microtubules (L. Wilson, personal communication) reveals that plant and animal tubulin are not identical; therefore, a chemical may interact with plant microtubular protein while having little or no affinity for animal tubulin. For example, it takes a greater concentration of colchicine to inhibit cell division in suspensions of plant cells than it does in suspensions of animal cells. In African blood lily liquid endosperm cells (plant cells with no cell walls), 10^{-4} M colchicine will block cell division (Hepler and Jackson, 1969), while in human HeLa cells the concentration required is 10^{-7} M (Taylor, 1965). In their report of electrophoretic difference between tubulin isolated from a plant and from an animal source, Olmsted et al. (1971) isolated tubulin from an alga (*Chlamydomonas*) and from brain tissue. Electrophoretic comparison of the two protein sources indicated that one protomer (subunit of the tubulin dimer) was common to both, while one from each was unique in its electrophoretic mobility. Because purified higher plant tubulin has not been tested for its interaction with trifluralin, it is possible that trifluralin has an action mechanism similar to that of colchicine (i.e., binding to microtubule subunits in such a manner that polymerization is prevented).

Loss of microtubules was probably not caused by a general deterioration of cell structures as a result of inhibition of cell division. Propham blocks cell division but does not cause disappearance of microtubules from root cells.

The disappearance of microtubules from root cells may result from inhibition of the synthesis of tubulin or dysfunction of the microtubular organizing center (MTOC). Trifluralin may act directly on the morphological unstructured MTOC of root-tip cells—i.e., endoplasmic reticulum (ER) membranes that appear to be involved in synthesis or transport of tubulin to and from site of polymerization. MTOC in higher plants appears to be a loosely organized aggregate of smooth ER cisternae in the polar regions of cells. Polar ends of the microtubules seem to terminate in membranes or vesicles of the ER elements (Hanzely and Schjeide, 1973). Burgess and Northcote (1968) reported that smooth ER was involved in polymerization and depolymerization of tubulin in wheat roots. Hanzely and Schjeide (1973) also found that smooth ER was present in the body of the spindle, as well as in polar regions. At both locations, various types of connections or attachments were observed between ER elements and microtubules. Figure 5-10 shows that ER of root cells from wheat and corn was swollen and distorted in trifluralin-treated roots, as compared to controls. If the proposed involvement of ER in microtubule assembly is correct, then an inhibition of the normal ER function, such as the observed swelling, could explain the loss of microtubules from the treated root cells. Additional support comes from the work of Towne (1974), who found that trifluralin and orzyalin (3,5-dinitro-N^4,N^4-dipropylsulfanilamide) herbicides caused leakage of photosynthetic products from single isolated cells, indicating that cellular membranes were altered or modified by the herbicides. Figure 5-9 shows that treated cells have enlarged vacuoles and that the root cells enlarge radially, suggesting that the selective permeability of cellular membranes may have been affected. Partitioning into and binding of trifluralin to the membranes could alter their activity.

Because trifluralin inhibits photosynthesis and respiration (Moreland et al., 1972), the herbicides conceivably could interfere with protein synthesis through their effects on the availability or formation of energy-rich compounds, such as ATP needed for protein synthesis and GTP necessary for polymerization of microtubular subunits into microtubules (Borisy and Olmsted, 1972). However, Moreland et al. (1972) reported that respiratory inhibitors and uncouplers do not usually produce the multinucleate cells or promote the radial enlargement of tissue that are typical plant responses to trifluralin and to colchicine.

SUMMARY

The mode of action of trifluralin is still unknown. Whatever the process may be, trifluralin achieves its effect rapidly (within 3 hours) and at low concentrations (less than 3×10^{-6} M). The anatomical effect of trifluralin treatment is aberrant cell division and cell enlargement in root tips. The cytological effect is the disappearance of microtubules from these areas. Our results suggest that the primary action mechanism of trifluralin may be related to an interference with one or more of the following: (1) the synthesis of tubulin; (2) the polymerization of microtubular subunits through action on the MTOC; or (3) the polymerization of microtubular subunits by interaction with the subunits themselves. Trifluralin's primary mechanism of action, however, remains to be explained.

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