Photosynthesis Is Not Involved in the Mechanism of Action of Acifluorfen in Cucumber (*Cucumis sativus* L.)

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ABSTRACT

The possible role of photosynthesis in the mechanism of action of the herbicide acifluorfen (2-chloro-4-(trifluoromethyl)phenoxy-2-nitrobenzoate; AF) was examined. The sensitivity to AF of cotyledons of cucumber (*Cucumis sativus* L.) which had been grown under far red light (FR) and white light were compared. FR grown tissues which were photosynthetically incompetent were hypersensitive to AF under white light and had approximately the same relative response to AF under blue and red light as green, white-light-grown tissues. Ultrastructural damage was apparent in FR-grown, AF-treated tissues within an hour after exposure to white light, with cytoplasmic and plastidic disorganization occurring simultaneously. In cucumber cotyledon tissue which had been greening for various time periods, there was no correlation between photosynthetic capacity and herbicidal efficacy of AF. PSII inhibitors (atrazine and DCMU) and the photophosphorylation inhibitor, tentoxin, had no effect on AF activity. Atrazine did not reduce AF activity at any concentration or light intensity tested, indicating that there is no second, photosynthetic-dependent mechanism of action operating at low AF concentrations or low fluence rates. Carbon dioxide-dependent O_2 evolution of intact chloroplasts of spinach (*Spinacia oleracea* L.) had an AF I_{50} of 125 micromolar compared to 1000 micromolar for cucumber, whereas AF was much more herbicidally active in tissues of cucumber than of spinach. Differences in activity could not be accounted for by differences in uptake of AF. Our results indicate that there is no photosynthetic involvement in the mechanism of action of AF in cucumber.

The *p*-nitro substituted DPE[2] herbicides typically cause rapid chlorosis (bleaching) by triggering oxidative destruction of both Chl and carotenoids (16, 19, 25). Their activity is expressed only in light (19, 25, 31) and their gross effects are much like those of bipyridinium herbicides such as paraquat (19). The role of light in activity of DPE herbicides is unclear, with evidence of photosynthetic involvement and of involvement of only carotenoids as photoreceptors.

Matsunaka (20) found nitrofen, a *p*-nitro DPE, to be equally effective on yellow mutants and the green wild type of rice. Albino rice seedlings were insensitive to nitrofen. These results led Matsunaka to conclude that carotenoids were the photoreceptors for nitrofen activity. Similar results with *p*-nitro DPE have subsequently been obtained with Chl and carotenoid mutants of other species (11, 20) and by using herbicides or phyto-

toxins which inhibit Chl or carotenoid synthesis (5, 7, 21). A crude action spectrum of the effect of oxyfluorfen on buckwheat leaves indicated activity in the blue portion of the spectrum with no activity in the red (31). Inhibition of photosynthetic electron transport has little or no effect on efficacy of these herbicides in higher plant systems (7) and some algal systems (9). Furthermore, acifluorfen methyl did not cause lipid peroxidation of photosynthetically active thylakoid membranes in which diuron and paraquat caused rapid peroxidation of membrane constituents (9). There is evidence that DPE herbicides can act as photosensitizers *in vitro*, in the absence of photosynthetic membranes to enhance the production of free radicals (26, 27); however, formation of DPE free radicals is not necessary for herbicidal activity (10).

Although these data clearly indicate that photosynthesis is not involved in the mechanism of DPE action, considerable evidence to the contrary has also been published (3). Photosynthetic electron transport is inhibited by *p*-nitro DPE such as nitrofen and oxyfluorfen at concentrations which are considerably higher than herbicidal levels (4, 22, 28, 30). Red light is very effective in algal systems in photosensitizing the herbicide (19). Lipid peroxidation caused by DPEs in certain green algae species can be prevented by photosynthetic electron transport inhibitors (17). Furthermore, DPE-caused radical formation in spinach chloroplast supsensions, as detected by electron spin resonance procedures, can be prevented by DCMU (18). The ESR spectra of paraquat- and DPE-generated radicals, however, differed. Phytotoxic symptoms caused by DPE in higher plants have been reported to be delayed until concurrently applied PSII inhibitors were metabolized by the plant (3), although this phenomenon has not been rigorously established. Green, but photosynthetically inactive cell cultures of spinach (3) and soybeans (RL Meeusen, personal communication) are insensitive to acifluorfen. In the green alga *Scenedesmus*, carotenoid content did not influence oxyfluorfen activity as long as photosynthetic electron transport remained operative (17). In intact chloroplasts of higher plants, CO_2-dependent O_2 evolution is rapidly affected by DPE and the effect was pronounced under red light (1). More recently DPEs have been shown to inhibit ferredoxin-thioredoxin reductase-dependent enzymes of carbon fixation (32).

Thus, the question of photosynthetic involvement in DPE mechanism of action is still open, with considerable conflicting evidence. In this paper we present further evidence in support of the view that photosynthesis is not directly involved in the mechanism of action of *p*-nitro DPE in higher plants.

MATERIALS AND METHODS

Plant Material. Cucumber seeds (*Cucumis sativus* L. [cv Straight Eight]) were planted in flats in a commercial greenhouse substrate (Jiffy-mix; JPA, West Chicago, IL) and watered with distilled H_2O. Plants were grown at 25°C for 6 to 8 d under 150

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[2] Abbreviations: DPE, diphenyl ether; AF, acifluorfen; AFM, acifluorfen-methyl; FR, far red light.

μmol·m^{-2}·s^{-1} PAR white light and >90% RH before cotyledons were harvested for use. In other experiments, plants were grown under a previously described FR source (6) until 6 to 8 d old, when cotyledons were harvested for experiments. In the different greening experiments, plants were grown in complete darkness for 5 to 7 d, at which time they were exposed to 150 μmol·m^{-2}·s^{-1} PAR of white light for various time periods (up to 24 h) and returned to darkness. Broad band red and blue lights were produced by filtering light from a GE cool beam 150 W incandescent lamp through 20 cm of water and either a red (No. 823) Roscolene cinemoid filter or a blue Corning glass (No. 5031) filter.

Electrolyte Leakage. Electrolyte leakage was assayed as before (7, 15) by cutting 50, 4-mm diameter cucumber cotyledon or market spinach leaf discs with a cork borer and washing them in 1% sucrose, 1 mM Mes (pH 6.5) and then placing them in a 6-cm diameter polystyrene Petri dish with 5 ml of the wash medium with or without herbicide. Technical grade herbicides were used: acifluorfen, gift of Rohm and Haas Co.; atrazine, gift of Ciba-Geigy Corp.; DCMU, Polyscience Inc.; and tentoxin, Sigma Chemical Co. The discs were then incubated at 25°C in darkness with or without the herbicide for 18 to 20 h, after which time the dishes were exposed to light of various qualities and fluence rates (see above). At the beginning of the light period and periodically thereafter conductivity of the bathing medium was measured with a conductivity meter. Electrolyte leakage experiments were repeated 2 to 10 times. Within each experiment, each treatment was triplicated. Results shown are means of triplicate samples from representative experiments.

Ultrastructure. The edges of cotyledon discs were cut away and the inner 1 mm^2 of the discs were fixed, embedded, sectioned, and stained as before (15). Electron micrographs were taken with a Zeiss EM10CR electron microscope. Multiple samples were prepared and examined at all sampling periods.

Photosynthetic Rates of Leaf Discs. CO$_2$-dependent O$_2$ evolution was measured polarographically by placing five cotyledon discs into either 0.5 or 1% sucrose, 12 mM Hepes-NaOH (pH 7.6), and 20 mM NaHCO$_3$ at 25°C without herbicide in a Hansatech DWI O$_2$ electrode unit (Hansatech, Ltd., Norfolk, England). Light intensity was 500 μmol·m^{-2}·s^{-1} PAR. Respiration was measured as O$_2$ consumption in darkness. The rate of O$_2$ consumption by respiration was assumed to be equal in light and dark in order to calculate a net rate of CO$_2$-dependent O$_2$ evolution. This experiment was repeated three times.

Effects of AF on CO$_2$-Dependent O$_2$ Evolution of Chloroplasts. Market spinach or 8- to 10-d-old cucumber cotyledons were ground (100 g in 500 ml) in 0.33 M sucrose, 30 mM Tricine-NaOH, 2 mM EDTA, 0.1% BSA (fatty acid free), 0.2% isoascorbate at pH 7.8 and 0°C in a blender set at full speed in two, 2-s bursts. The brei was filtered through four layers of cheesecloth and two layers of Miracloth. The filtrate was then centrifuged at 1500g for 5 min. The pellet was gently resuspended with a sable artist's brush in 2 to 3 ml of 0.33 M sorbital, 50 mM Hepes-NaOH, 1 mM MgCl$_2$, 2 mM EDTA, and 10 mM NaHCO$_3$ at pH 7.6 and 2°C. The chloroplast suspension was loaded onto a precentrifuged 50% v/v Percoll gradient made up in the same buffer and centrifuged for 10 min at 5000g. Intact chloroplasts were recovered, washed in 25 ml of resuspension buffer, centrifuged at 270g for 5 min, and resuspended in a minimal volume of resuspension buffer. CO$_2$-dependent O$_2$ evolution was measured in a buffer solution of 50 mM Hepes-NaOH, 0.33 M sorbitol, 2 mM EDTA, 10 mM NaHCO$_3$, 10 mM KCl, and 5 mM Na$_4$P$_2$O$_7$ at pH 7.6 and 25°C with a Hansatech DWI O$_2$ electrode unit. A second reaction mixture was used with 20 μg of Chl per ml. Chloroplasts from both species were 90% or more intact as measured by ferricyanide-dependent O$_2$ evolution. Light intensity was approximately 500 μmol·m^{-2}·s^{-1} PAR, provided by a

GE cool-beam flood lamp. This experiment was repeated three times.

Chlorophyll Content. Chl was assayed by the method of Hiscox and Israelstam (12), by steeping leaf or cotyledon discs in 5 ml of hot (60°C) DMSO for 30 min, centrifuging the extraction liquid at 5000g for 15 min, and then spectrophotometrically determining Chl content.

[^{14}C]AF Absorption. Leaf or cotyledon discs of spinach and cucumber, respectively, were incubated as above in 5 ml of buffer with 9.5 nCi of uniformly labeled [^{14}C]AF. The specific activity was such that the concentration of AF in the Petri dishes was 27 μM. At time points during dark preincubation, five discs per dish were removed, washed with distilled H$_2$O three times, and frozen in paper combustion cones. Samples were combusted and resulting CO$_2$ trapped in a commercial CO$_2$-trapping fluid. Sample ^{14}C content was measured with scintillation spectroscopy, correcting for background and quenching. This experiment was repeated three times and all treatments were triplicated in each experiment. Results from a typical experiment are given.

RESULTS

Effects of AF on Tissues Grown Under FR Light. Plants grown under continuous FR had no detectable CO$_2$-dependent O$_2$ evolution (data not shown). FR-grown tissues were more sensitive to AF when exposed to white light than was green tissue (Fig. 1A). In both green and FR-grown tissues leakage was almost linear in acifluorfen treatments for 8 h, after which the leakage rate began to slow. The differences in leakage were not due to
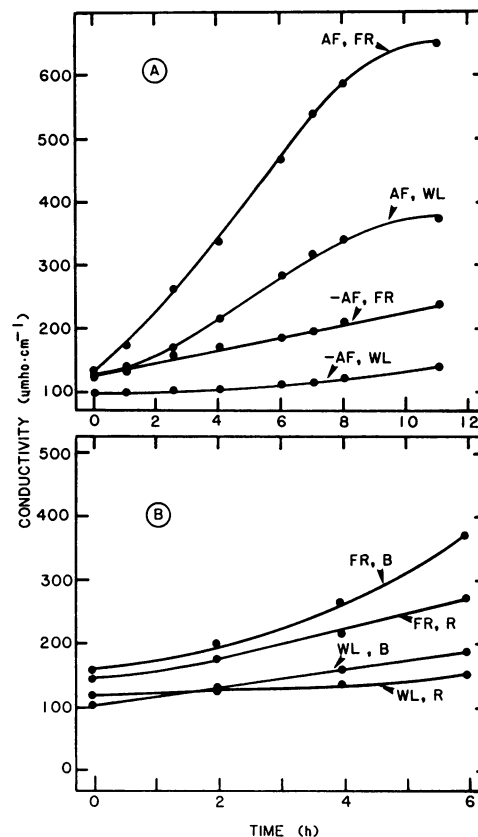

FIG. 1. Electrolyte leakage from FR-grown (FR) and white-light grown (WL) cucumber leaf discs incubated for 20 h in darkness with or without 30 μM AF. After the dark incubation, light-induced electrolyte leakage was measured under exposure to (A) white light (400 μmol·m^{-2}·s^{-1} PAR) or (B) blue (B) and red (R) light, each at 180 μmol·m^{-2}·s^{-1} PAR.

differences in potential electrolyte leakage. At the end of the dark incubation period (*i.e.* at the time they would be transferred to the light), the discs were homogenized in the buffer solution in which they were incubated. Conductivities of supernatants of homogenates of discs from FR-grown and white light-grown plants were 582 and 520 μmho, respectively.

The relative degree of leakage of herbicide-treated tissues under blue light when compared to red light was similar in FR-grown and white light-grown tissues (Fig. 1B), although the FR-grown tissues were more sensitive to the herbicide.

Plastids of FR-grown plants looked the same after the 20 h dark period, whether treated with AF or not (Fig. 2A). The plastids were very similar to etioplasts, with prolamellar bodies, nonstacked prothylakoids, and a few stacked thylakoids. Within 1 h after exposure to white light, a variety of ultrastructural damage was observable in AF-treated tissues. Cells were observed with unaffected plastids, but with disrupted tonoplasts and/or plasmalemmas (not shown). Other cells had disrupted tonoplasts and swollen, vesiculated plastids (Fig. 2B). By 2 h there were many cells in which the tonoplast and/or plasmalemma were broken and the plastids were filled with vesicles, starch grains, and remnants of prolamellar bodies (Fig. 2C). Generally, the ultrastructure of all cytoplasmic contents, including the plastid, plasmalemma, and tonoplast was disrupted almost simultaneously. FR-grown tissues that were not treated with AF were not significantly different after 2 h than before exposure to white light (Fig. 2D).

Effects on Tissues at Various Stages of Greening. Little or no correlation was observed between the capacity for CO_2-dependent O_2 evolution at the beginning of exposure to white light and the efficacy of AF in causing leakage of electrolytes from tissues after 3 h of light exposure (Fig. 3). Similar results were obtained when leakage at different times after light exposure were plotted (data not shown). CO_2-dependent O_2 evolution was lost in all treatments within 3 h of light exposure after the 20 h dark incubation (data not shown), as before (15). Leakage of etiolated tissues was approximately the same as from tissue that had been greened for 24 h and had become fully photosynthetically competent.

PSII Inhibitor-Treated Tissues. PSII inhibitors (atrazine and DCMU) had no antagonistic effects on AF's action in causing cell leakage (Fig. 4). AF plus atrazine usually caused greater and faster leakage than the AF alone. Tentoxin, an inhibitor of photophosphorylation (2) by inhibition of CF_1 ATPase (29), also had no effect on AF activity (Fig. 4). Each of these inhibitors was demonstrated to have been absorbed by the cotyledon discs before exposure to white light by their effects on variable fluorescence transients (Fig. 4, inset). Tentoxin at 10 μM greatly reduced variable fluorescence, as an inhibitor of the oxidizing side of PSII would be expected to do. At 1 μM tentoxin had no effect on F_{max} but slowed the rapid decay from F_{max} that normally occurs within 5 to 8 s (data not shown).

At a range of concentrations (1–33 μM) of AF, in high and low light, atrazine had no effect or slightly increased AF activity (Fig. 5), demonstrating that there is no involvement of PSII in AF action at a range of photosynthetic or AF activities. Similar results were obtained with AFM (data not shown).

Effects of AF on Photosynthesis of Isolated Chloroplasts. AF inhibited CO_2-dependent O_2 evolution in spinach much more effectively than that in cucumber with I_{50}s of approximately 125 and 1000 μM, respectively (Fig. 6). In contrast, discs of cucumber cotyledons were affected much more by AF than spinach leaf discs, as measured by cellular leakage and pigment bleaching (Fig. 7). This difference could not be accounted for by differences in AF uptake. After the 20 h dark incubation period there was an approximate 2-fold higher uptake of ^{14}C-AF by cucumber than by spinach.on a fresh weight basis (239 ± 28 *versus* 106 ±

8 dpm/mg). Based on the water content of the tissues, the AF concentration in cucumber and spinach were 166 and 78 μM, respectively. Considering the incubation buffer contained 27 μM AF, both tissues accumulated AF to higher concentrations than in the medium. After exposure to 400 μmol·m^{-2}·s^{-1} PAR white light, the cucumber tissues lost ^{14}C, whereas the spinach continued to take up [^{14}C]AF, resulting in an AF concentration of 114 μM in spinach tissue 9 h after exposure to light. The 2-fold higher concentration of AF in cucumber than in spinach might partly explain the greater sensitivity of cucumber to AF; however, the large difference in sensitivity could not be explained by this finding.

DISCUSSION

All of the evidence presented in this paper is consistent with the hypothesis that there is no photosynthetic involvement in the mechanism of herbicidal action of AF.

The results with FR-grown tissues were similar to those obtained previously with tentoxin-treated plants (7). Treatment with tentoxin, a fungal toxin, causes a yellow, chlorotic plant that is phenotypically similar to a FR-grown plant. In both cases the chlorotic, photosynthetically nonfunctional tissue is hypersensitive to the herbicide. This result may be due to a lack of ability to regenerate protective compounds which are consumed by peroxidative reactions. For instance, although there was not a difference in the level of ascorbate in green and FR-grown cucumber cotyledon discs, ascorbate was much more rapidly consumed in FR-grown than green tissues treated with AF (14).

The finding that the relative effects of broad band blue and red light were similar in FR-grown and green tissues indicates that the red light effect on AF efficacy in green tissues is independent of photosynthetic activity. This conclusion is in agreement with the finding of Ensminger and Hess (8) that there is a major peak in the blue and a lesser one in the red in the action spectrum of AFM activity on *Chlamydomonas eugametos*. They concluded, however, that the effect was not due to photosynthesis, since inhibitors of both cyclic (24) and noncyclic (9) photosynthetic electron transport do not affect AFM activity. Furthermore, they concluded that if the red peak were due to Chl acting as accessory pigments for carotenoid-initiated phytotoxic events, photosynthetic electron transport inhibitors should enhance DPE activity. Thus, our results with FR-grown tissues and the conclusion of Ensminger and Hess (8) indicate that the red light-induced activity is due to a Chl pigment which is not involved in photosynthesis or to a red-light-absorbing chemical or exciplex (charge-transfer complex) unrelated to Chl.

The time course for disruption of cellular ultrastructure of FR-grown tissues (Fig. 2) was also much faster than that previously observed with green tissues (15). In a previous study (15) the tonoplast and plasmalemma appeared to be the first major membrane systems to be disrupted by AF, while the chloroplast envelope and thylakoids were the last membranes to be disrupted. The sequence of events in FR-grown tissues was not as clear, however. There may be two reasons for this: (a) the much more rapid development of effects, and (b) the inability of the plastids of FR-grown plants to photosynthetically regenerate protective compounds such as ascorbate, reduced glutathione, and α-tocopherol.

The lack of correlation of photosynthetic competence with sensitivity to AF (Fig. 3) also indicated that photosynthetic capacity is not a prerequisite for AF activity. Tissues were about equally sensitive to the herbicide, regardless of degree of chloroplast development. Orr and Hess (23) found partially developed cucumber cotyledons to be more susceptible to AFM damage than tissues that were greened for a longer period. No determination of photosynthetic activities were made in their study, however.
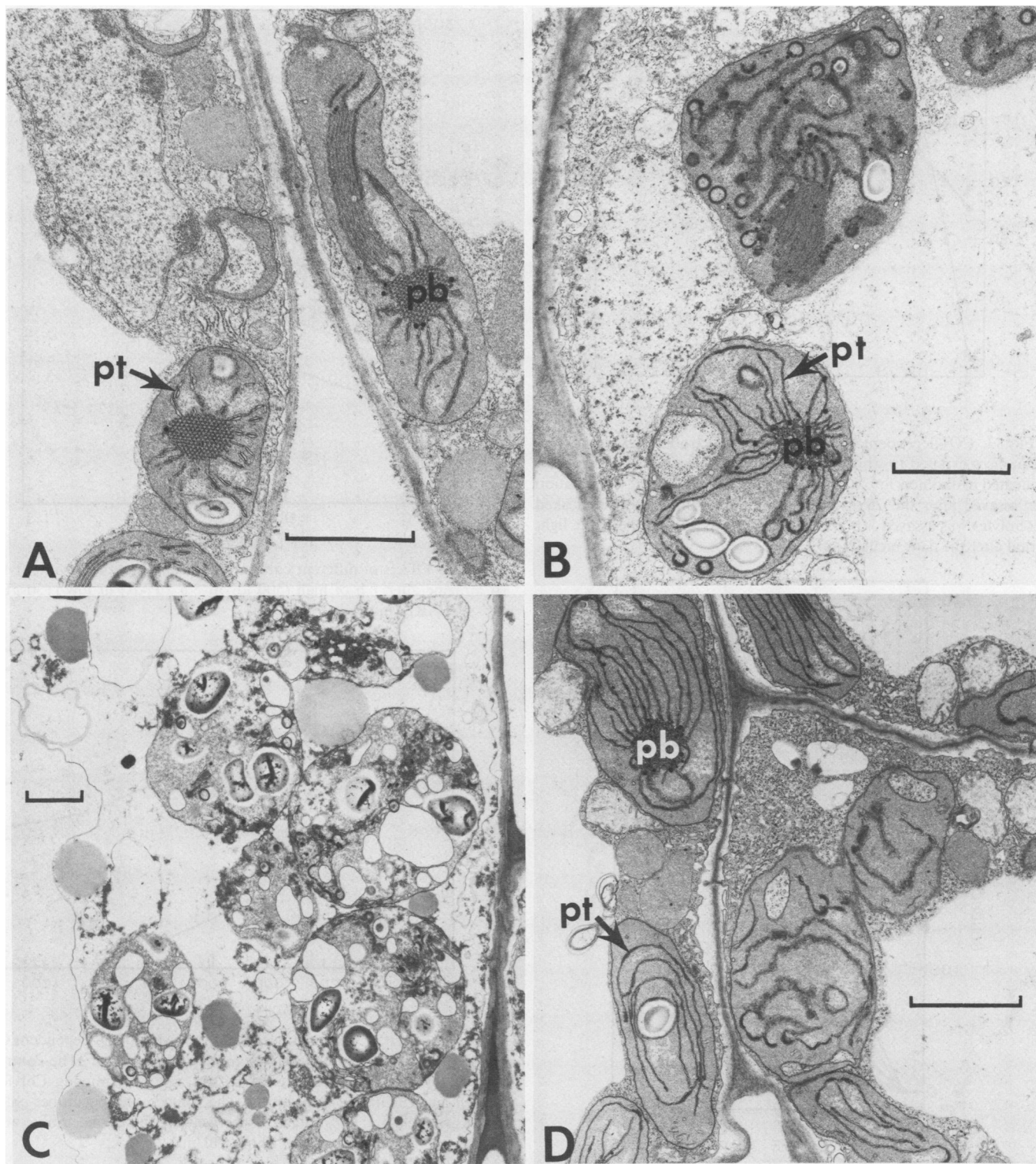
FIG. 2. Ultrastructural development of herbicide symptoms in FR-grown cucumber cotyledon discs treated with 30 μM AF. A, Appearance of cells after 20 h dark period at the beginning of the exposure to white light; B, acifluorfen-treated tissue after 1 h of exposure to white light; C, acifluorfen-treated tissue after 2 h exposure to white light; D, control (not herbicide treated) FR-grown tissue after 2 h of white light. Bars = 1 μm, except in B where the bar is 2 μm, pb = prolamellar body, pt = prothylakoids.

The lack of effect of PSII electron transport inhibitors at various light intensities and AF concentrations (Figs. 4 and 5) indicates that in AF-treated cucumber there is no requirement for photosynthetically derived reducing power in order to generate herbicidal effects. Similar results have been reported by Ensminger and Hess (9) with diuron with AFM, oxyfluorfen,

and MC 15608 (methyl 5-[2-chloro-4-(trifluoromethyl) phenoxy]-2-chlorobenzoate) in *Chlamydomonas* cells. Matsunaka (20) found DCMU to have no effect on nitrofen activity in rice and our lab (6) and that of Orr and Hess (24) have found atrazine and DCMU to have no effect on DPE activity in cucumber cotyledons. The present work excludes the possibility that PSII
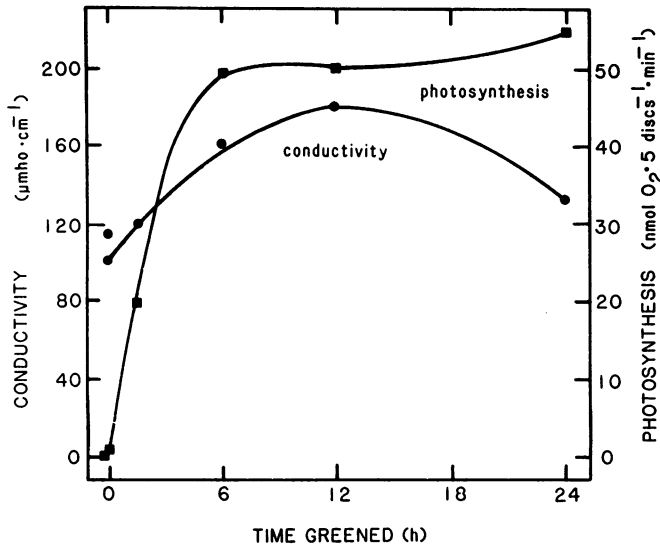
FIG. 3. CO_2-dependent O_2 evolution and electrolyte leakage of cotyledon discs exposed to white light for various periods (x axis) and then incubated in darkness for 20 h in 30 μM AF. CO_2 dependent O_2 evolution was assayed after the 20 h dark incubation. Conductivity of leaked electrolytes was assayed 3 h after beginning exposure to the white light period after the dark incubation.
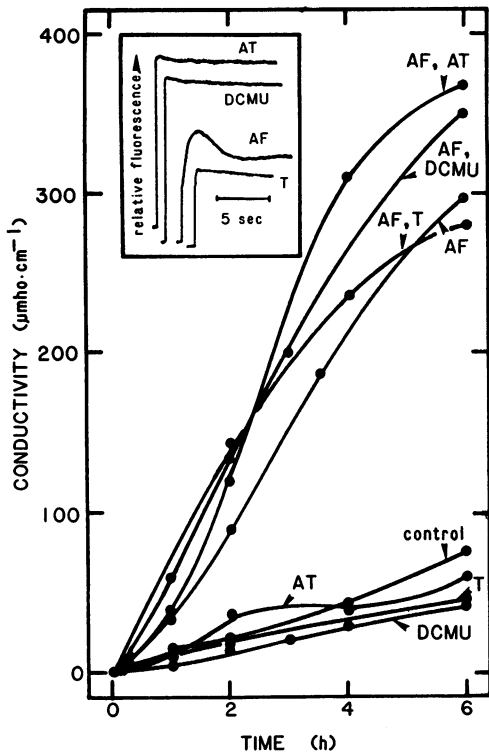

FIG. 5. Effects of different concentrations of AF with or without 10 μM atrazine after 7 h of high (500 μE·m^{-2}·s^{-1} PAR) or low (150 μE·m^{-2}·s^{-1} PAR) white light.

FIG. 4. Effects of atrazine (AT), DCMU, and tentoxin (T), each at 10 μM, on cellular leakage caused by AF. Effects under high light. Conductivity is presented as change from conductivity at the beginning of white light exposure. Inset, variable fluorescence before exposure to white light, used to measure effectiveness of photosynthetic inhibitors. AF-treated and control discs had identical variable fluorescence at this time.

is a requirement only at low DPE concentrations or low light intensities, i.e. a PSII-requiring mechanism which is swamped by a second, photosynthetically independent, mechanism at higher herbicide or light levels. In fact, the consistently slightly higher activity of AF in the presence of photosynthetic inhibitors
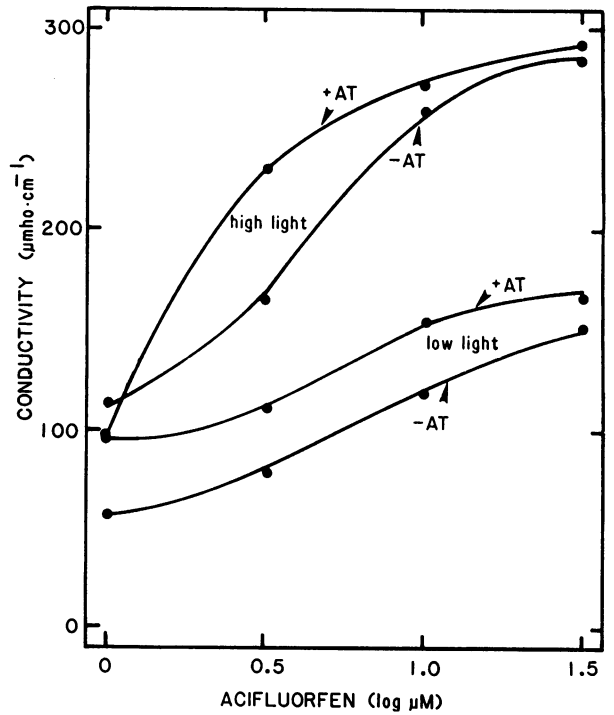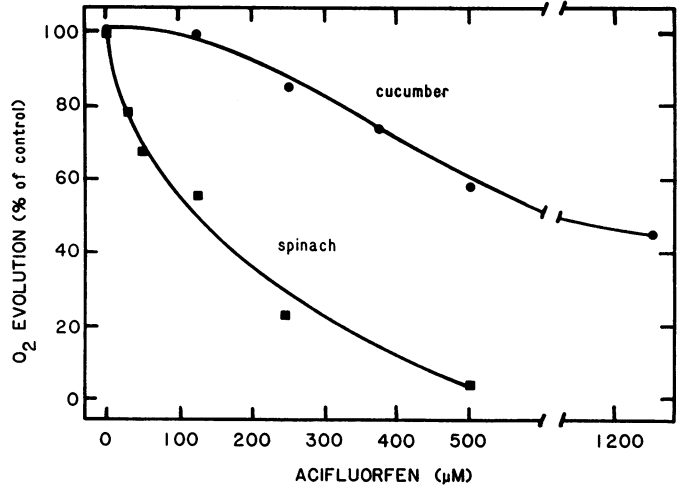

FIG. 6. Effects of various concentrations of AF on CO_2-dependent O_2 evolution of intact chloroplasts of spinach and cucumber. The control rates of O_2 evolution were 1.67 and 0.14 nmol O_2/min·μg Chl for spinach and cucumber, respectively. The spinach and cucumber chloroplasts were 90 and 98% intact, respectively. With both cucumber and spinach preparations, there was 10 mg Chl/ml.

indicates that photosynthesis provides some protection against DPE—probably through regeneration of antioxidants (14).

Experiments from Böger's laboratory with diuron and oxyfluorfen in *Scenedesmus* (16) and *Bumilleriopus* (19) have given completely different results, i.e. reversal of the DPE effect with diuron. In these species, DPEs may not have the same mechanism of action as in higher plants. Also, oxyfluorfen may act at a different site than AF, because of its greater lipophilicity. We found, however, that atrazine had no effect on activity of AFM a more lipophilic and more active DPE than AF.

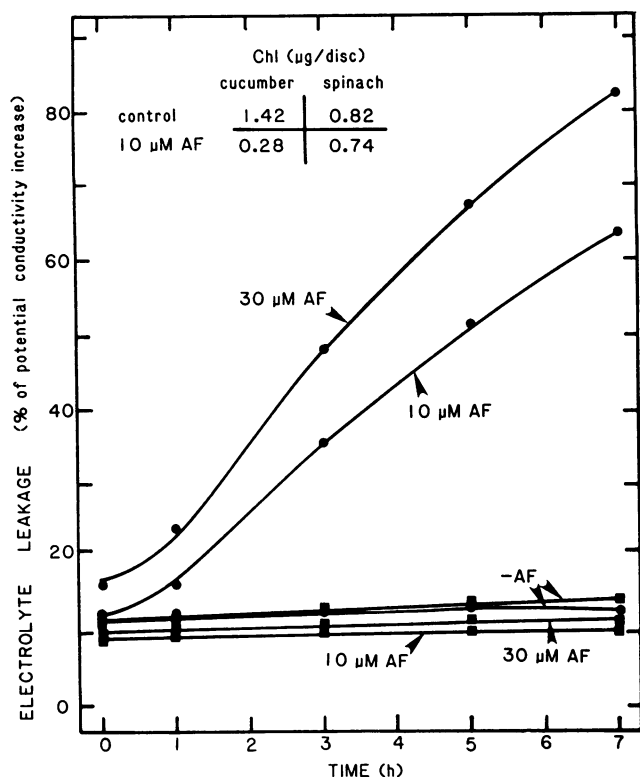Results with tentoxin (Fig. 4) indicate that photophospho-

FIG. 7. Effects of AF on cellular leakage and Chl content (after 24 h of light) of spinach leaf (■) and cucumber cotyledon (●) discs. For comparison purposes data are shown as percent of potential leakage. Inset, Chl content after 24 h of light.

rylation is not required for AF activity. Although the effect of 10 μM tentoxin on the fluorescence transient indicated that the oxidizing side of PSII was inactivated, 1 μM treatment (not shown) caused a reduction of the rate of loss in variable fluorescence after attaining F_{max}. This loss of variable fluorescence is thought to be due to the phosphorylation of light-harvesting Chl proteins associated with State 1-State 2 transitions (13). Thus, at 10 μM, photophosphorylation and electron flow were probably both halted. This is in agreement with Arntzen's (2) finding that tentoxin inhibits both photophosphorylation and, at higher concentrations, flow of electrons to PSI.

We found that the relative efficacy of AF on isolated chloroplasts of spinach and cucumber are opposite to the relative efficacy of the intact tissues. Inhibition of CO_2-dependent O_2 evolution by AF was found to require considerably higher herbicide concentrations than are required for herbicidal effects on intact tissues. These findings support the hypothesis that effects on photosynthesis are secondary to the as yet unknown primary effect of AF. Others have indicated that AF inhibition of CO_2-dependent O_2 evolution in spinach is due to inhibition of light activation of fructose 1,6-bisP and glyceraldehyde-3-P dehydrogenase, probably through interference with the plastid Fd/Fd-thioredoxin reductase/thioredoxin system (1, 32). If this effect were the primary herbicidal site of AF, a sequence of secondary events much like that of slow paraquat damage or a runaway Mehler reaction would be expected. This is not the case (9, 15). Evidence of superoxide and/or H_2O_2 formation in the plastid due to reduction of molecular O_2 to superoxide radical and subsequent dismutation to H_2O_2 would be observed if carbon fixation were blocked. Neither superoxide radical nor H_2O_2 have been detected in situ as a result of DPE action (7, 32). In broken chloroplasts, p-nitro substituted DPEs do stimulate Mehler reaction; however, in situ p-chloro substituted DPEs are equally herbicidal, although they have no effect on the Mehler reaction

(28). Furthermore, copper penicillamine complex, an artificial superoxide dismutase, provides excellent protection against paraquat damage, while providing little (7) or no (9) protection against DPE. The differences in sensitivity of intact cells of spinach and cucumber to AF may be due to differences in levels of protective systems.

Our results are strong evidence that AF does not require photosynthetic activity for light-mediated herbicidal action. Nor, apparently, is its interference with photosynthetic activity in situ directly involved in the peroxidative damage observed. Our findings support the view that AF exerts its effects through a carotenoid-mediated photosensitizing reaction (25, 27).

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