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# Influence of Chloroplast Development on the Activation of the Diphenyl Ether Herbicide Acifluorfen-Methyl

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## ABSTRACT

**The activity of acifluorfen-methyl (AFM); methyl 5-(2-chloro-4-[trifluoromethyl] phenoxy)-2-nitrobenzoate in excised cucumber cotyledons (*Cucumis sativus* L.) was examined. AFM induced membrane disruption, was significantly greater when etiolated cotyledons were illuminated 16 hours at 150 microeinsteins per square meter per second photosynthetically active radiation *versus* incubation under illumination of 4-fold greater intensity. These results were unexpected since the loss of membrane integrity is initiated by photodynamic reactions. Untreated, etiolated cotyledons were not able to accumulate chlorophyll under the higher light intensity while control and herbicide treated cotyledons greened significantly under the lower intensity illumination suggesting that some process associated with greening stimulated AFM activity. Inhibition of greening by cycloheximide also reduced AFM activity. Intermittent lighting induced greening in AFM treated cotyledons without causing any detectable loss of plasmalemma integrity. Utilization of this system for pretreatment of cotyledons prior to continuous illumination revealed that activity was greater when tissue was greened in the presence of AFM than when herbicide treatments were made after a greening period of the same duration. The results indicate that the pigments *in situ* in etiolated tissue are sufficient, without greening, to initiate membrane disruption by AFM. However, greening increases the herbicidal efficacy greatly. Furthermore, the stimulation appears to be due to specific interactions between AFM and the developing plastid and is not attributable solely to an increase in endogenous photosensitizers.**

Since the initial work of Orr and Hess (19), which focused attention on the membrane disrupting effects of the 4′-nitro DPE[1] herbicides, many aspects of their mode of action have been well established. Reminiscent of the contact foliar bleaching and desiccation of bipyridilium herbicides such as paraquat, phytotoxicity is induced by light and oxygen dependent lipid peroxidation reactions (4, 9, 10, 19, 20). However, the mechanism of activation, that is those processes which initiate the generation of oxygen radicals, is less well understood, but clearly differs fundamentally from the activation of the bipyridiliums. Etiolated tissues respond to the DPEs and inhibition of photosynthetic electron flow has been reported not to block DPE activity in higher plants (4, 5, 15, 20, 24). In isolated chloroplasts, the propensity of 4′-nitro DPEs for stimulating a Mehler reaction was found to differ significantly from the responses to paraquat (25). Furthermore, 4′-Cl analogs which do not appear capable of reduction by biologically generated potentials, are also effective membrane disruptors (21, 22, 25).

[1] Abbreviations: DPE, diphenyl ether; AFM, acifluorfen-methyl; LHCP, light harvesting complex protein.

Thus, the familiar model for paraquat activation, that of an autooxidizing, electron acceptor reduced by photosynthetic potentials, appears to have little application to the action of DPE herbicides in higher plants. The results of our studies on factors influencing the activity of AFM support the conclusions of earlier investigations that photosynthetic electron flow is not required to initiate DPE mediated lipid peroxidation and that the pigments *in situ*, in etiolated tissue, probably the carotenes, are sufficient to activate these herbicides and bring about significant membrane disruption (4, 7, 15, 20). However, our experiments also indicate a previously unappreciated role for biosynthetic processes in the full optimization of the herbicidal activity. In agreement with the action spectrum for AFM previously disclosed by Ensminger and Hess (6), we have seen evidence for an important role for Chl and/or Chl precursors in the activation process. Our experimental results suggest that even more important than an additive contribution by Chl towards the pool of available photosensitizing pigments, are interactions that take place between the developing plastid and the herbicide, synchronous with greening, that results in the formation of a significantly more efficient oxygen radical generator than occurs in systems where pigment synthesis is inhibited, or has been completed.

## MATERIALS AND METHODS

**Plant Material.** Cucumber seed (*Cucumis sativus* L. cultivar 'Wisconsin SMR 18') was germinated and grown in vermiculite irrigated with a commercial (9-45-15) fertilizer. Seedlings were grown at 25°C and 80 to 90% RH in a dark incubation chamber. Cotyledons were harvested from the etiolated seedlings 5 d after planting and were rinsed in 1.0 mM $CaCl_2$.

**Efflux Experiments.** Determinations of the extent of disruption to the plasmalemma were made according to a modification of the procedure of Orr *et al.* (21). For each experiment, 180 to 230 washed cotyledons were added to a 250 ml widemouth, foam-stoppered, Erlenmeyer flask containing 50 ml of an uptake media consisting of 1 mM KCl, 1 mM $CaCl_2$, and 2.0 mM K-phosphate adjusted to pH 6.5. Uptake of the radiolabeled, efflux tracer was initiated by adding 3-O-methyl-D-[U-$^{14}$C]glucose of specific radioactivity 10.9 GBq/mmol (Amersham Corp.), to a final concentration of 600 nM in the uptake media. The flask was shaken for 24 h at 125 rpm on a gyrotory shaker in a dark incubation chamber at 25°C. The cotyledons were then recovered on a nylon mesh and rinsed three times in 20 ml volumes of 1 mM $CaCl_2$. The total uptake of radiolabeled sugar by the cotyledons (total potential efflux) was determined by digesting three samples of five cotyledons each, in NCS tissue solubilizer (Amersham Corp.) and counting the resulting macerate in a liquid scintillation spectrometer. Results from these digestions were found to be equivalent to the same determinations made by combusting sampled cotyledons in an autooxidizer. Treatments were made by floating the cotyledons, abaxial side up, on a buffer identical to the uptake media. Additions of AFM were made

from acetone stocks to a final solvent concentration of 0.1% (v/v). Controls were treated with identical concentrations of acetone. Throughout periods of dark or illuminated incubations of the treated tissue, the floating cotyledons were swirled by shaking the dishes at 90 rpm on the surface of a gyrotory shaker. All operations after planting and up to initiation of the specified illumination regimes, were carried out in the dark or under green fluorescent safe-lights. Illumination designated 'low light' was provided by four GE F20T12-CW fluorescent lamps at a measured intensity of 150 $\mu E/m^{-2}\cdot s^{-1}$ (PAR). Illumination designated 'high light' was produced by four 400 W sodium halide lamps (Westinghouse LU400) in combination with four 400 W mercury vapor lamps (Westinghouse MVR 400/U) which delivered a measured intensity of 600 $\mu E/m^{-2}\cdot s^{-1}$ (PAR). Intermittent illumination, measured intensity 25 $\mu E/m^{-2}\cdot s^{-1}$ (PAR), was supplied by a single GE 'Bright Stick' controlled by an electronic timer (Chrontrol, Lindburg Enterprises, Inc. 4878 Ronson Ct., San Diego, CA). Following the specified period of illumination, effluxed radioactivity was determined by recovering all of the assay media that the cotyledons had floated on and counting these samples in a liquid scintillation spectrometer. The results are expressed as the Percent of Total Efflux, calculated by dividing the efflux level for each treatment by the mean total uptake value determined from the digested samples; thus, greater membrane damage is denoted by higher percentage values.

**Pigment Analyses.** Cotyledons were prepared and treated as in the efflux experiments except the addition of radiolabeled 3-O-methyl-D-[$U^{-14}$C]glucose was omitted. Following the specified illumination period, the five cotyledons from each treatment replicate were extracted in 5 ml of absolute methanol. The extracts were filtered and the pigment levels then calculated spectrophotometrically from the extinction coefficients of Lichtenthaler and Wellburn (12).

**Experimental Design.** All experiments were set out as completely randomized blocks with three replicates of each treatment and analyzed by a simple one-way analysis of variance made on combined experiments. Each experiment was conducted two to three times and all treatment values presented in figures and tables are the means from all replicates combined across experiments.

**Chemicals.** AFM was made in the synthesis laboratories at the FMC Chemical Research and Development Center, Princeton, NJ. Reagents were purchased from Aldrich of Sigma.

## RESULTS AND DISCUSSION

Investigations were begun on the response of AFM treated tissue under illumination of prolonged duration (16 h) where it was observed, unexpectedly, that the level of injury in etiolated cucumber cotyledons transferred directly into illumination of higher intensity was reduced relative to treatments incubated under illumination of a lower intensity (Fig. 1). In initial experiments, we found that the magnitude of the response under the lower intensity regime was quite uniform between experiments. But the level of activity observed under the high intensity regime, and thus the differential between the two conditions, showed a great deal of variation among individual experiments (data not shown). When all treatments and operations made prior to the exposure to continuous illumination were carried out in darkness, or under green safe-lights to suppress the initiation of pigment development prior to the beginning of the experiment, the response under both lighting regimes stabilized and the differential between them was maximized. Differences in the efflux response proved to be greatest at lower concentrations, 0.25 to 1.0 $\mu$M, at higher concentrations, 5 $\mu$M and above, the response curves under both illumination regimes began to plateau (Fig. 2).

The sensitivity of the high light intensity treatments to initial

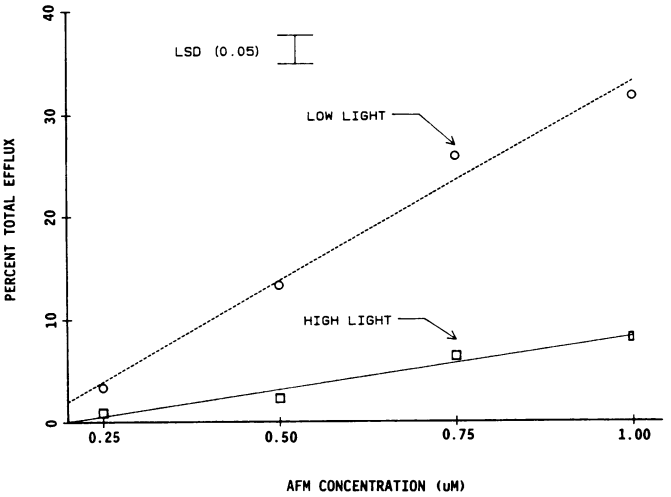


FIG. 1. AFM dose response as a function of light intensity. Under green safelights, excised cotyledons were placed on media treated with various concentrations of AFM then held in darkness for 6 h. Treatments were then placed under illumination of one of two intensities: 600 $\mu E/m^{-2}\cdot s^{-1}$; PAR (high light, □——□) or 150 $\mu E/m^{-2}\cdot s^{-1}$; PAR (low light, O——O). Following 16 h or continuous illumination the treatment media was recovered and sampled for effluxed 3-O-methyl-[U-$^{14}$C]glucose. Leakage induced by the herbicide treatments is expressed as a percent of the total uptake of radioactivity; therefore, increasing injury is expressed as an increase in the percentage value.

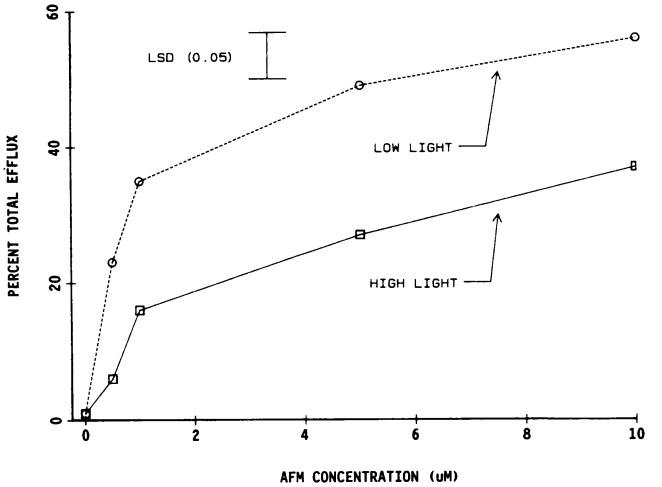


FIG. 2. AFM dose response as a function of light intensity. Under green safelights, excised cotyledons were placed on media treated with various concentrations of AFM then held in darkness for 6 h. Treatments were then placed under illumination of one of two intensities: 600 $\mu E/m^{-2}\cdot s^{-1}$; PAR (high light, □——□) or 150 $\mu E/m^{-2}\cdot s^{-1}$; PAR (low light, O——O). Following 16 h of continuous illumination the treatment media was recovered and sampled for effluxed 3-O-methyl-[U-$^{14}$C]glucose. Leakage induced by the herbicide treatments is expressed as a percent of the total uptake of radioactivity; therefore, increasing injury is expressed as an increase in the percentage value.

lighting effects seemed to suggest that some photoactivated process was critical in influencing later herbicidal activity. A factor potentially responsible for the differential in herbicidal activity between the two lighting conditions was indicated by time course studies of pigment development in control (not treated with herbicide) cotyledons from both illumination regimes. These experiments showed that while carotene levels remained fairly similar under either lighting intensity, Chl development was

markedly inhibited when etiolated cotyledons were transferred directly from darkness (or green safe-lights) to the higher intensity light (Fig. 3). Evidently Chl and/or its precursors were being photooxidized prior to being safely integrated in the developing lamellae under the more intense lighting. Another timecourse was then conducted under similar conditions, in which efflux of 3-O-methyl-D-[U-$^{14}$C]glucose from cotyledons treated with 5 $\mu$M AFM was measured. In agreement with the previous results of Orr and Hess (19) a significant level of membrane disruption was observed earlier under the higher light intensity; however, after the 5 h sampling point, herbicidal injury developed to a greater extent under the lower intensity illumination (Fig. 4). The corresponding effect of the same concentration of AFM on pigments was a reduction in rate of accumulation of Chl and carotene which seemed to accompany rather than precede membrane disruption (compare Figs. 3 and 5). However, Figure 5 indicates that Chl concentrations did reach significantly higher levels in the lower light intensity treatments before herbicidal bleaching began, suggesting that the greening of the tissue was correlated with the greater herbicidal effect that eventually developed under the lower intensity illumination. Such a conclusion seemed to be supported by the action spectra reported by Ensminger and Hess (6) which indicated a role for Chl in the activation of AFM.

Various reports have also suggested that more than photosynthetic pigments might be required to initiate membrane disruption by these compounds. One of the more frustrating anomalies of investigations of the DPE herbicides has been the failure of these compounds to readily stimulate appreciable lipid peroxidation in *in vitro* chloroplast systems despite the utilization of thylakoids isolated from fully greened leaves, which contain all the pigments heretofore considered necessary for photoactivation (7). Furthermore, we had observed that green oat protoplasts, which were bleached by treatments with paraquat, did not seem to be susceptible to AFM (data not shown).

One possible implication of these various experimental results, including our observations on the influence of light intensity, was that optimal DPE activity required greening, rather than simply green tissue. This led to attempts to examine the relation between the greening process in cucumber cotyledons and AFM activation, but such investigations were invariably confounded by the necessity of using light to trigger plastid development, which in turn, when supplied continuously even at very low intensities, would initiate lipid peroxidations destructive to the

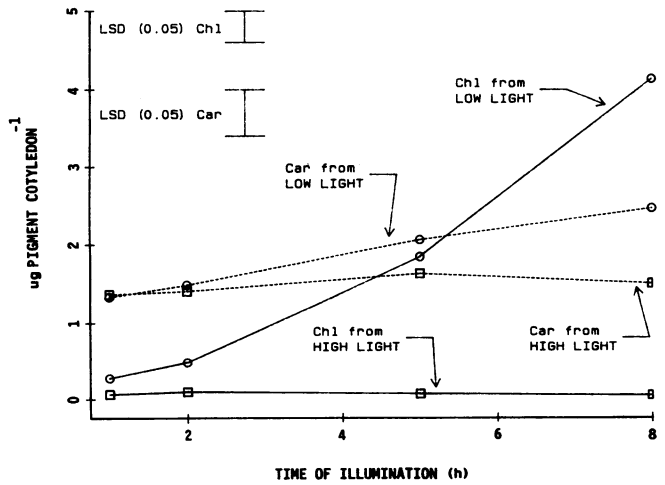


FIG. 3. Pigment accumulation in untreated cotyledons as a function of light intensity. Cotyledons were floated on untreated buffer, then placed under illumination of one of two intensities: 600 $\mu E/m^{-2}\cdot s^{-1}$; PAR (high light, □) or 150 $\mu E/m^{-2}\cdot s^{-1}$; PAR (low light, O) at time zero. Treatment sets of five cotyledons were sampled at the indicated time points and extracted for spectrophotometric pigment analysis. The levels of Chl (Chl ——) and carotenes (Car - - -) were determined by the method of Lichtenthaler and Wellburn (12).

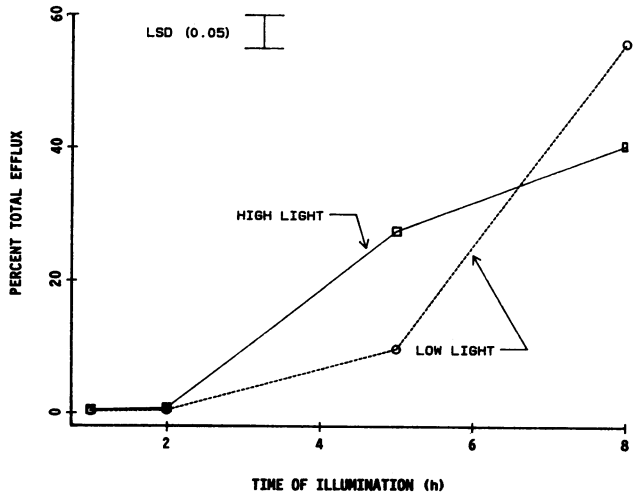


FIG. 4. Membrane damage in cotyledons treated with 5.0 $\mu$M AFM as a function of time and light intensity. Cotyledons were treated with 0.1% acetone (Controls) or 5 $\mu$M AFM, under green safelights, then incubated in a shaking flask in the dark for a 2 h uptake period. The treated tissue was washed in 1 mM $CaCl_2$, and floated on untreated buffer at time zero before being placed under continuous illumination so that treatment responses are not a reflection of continued herbicide uptake during the timecourse of the illumination period. Continuous light was supplied at one of two intensities: 600 $\mu E/m^{-2}\cdot s^{-1}$; PAR (high light, □——□) or 150 $\mu E/m^{-2}\cdot s^{-1}$; PAR (low light, O——O). At the time points indicated the extent of light activated membrane disruption was determined by sampling the treatment media for effluxed 3-O-methyl-[U-$^{14}$C] glucose.

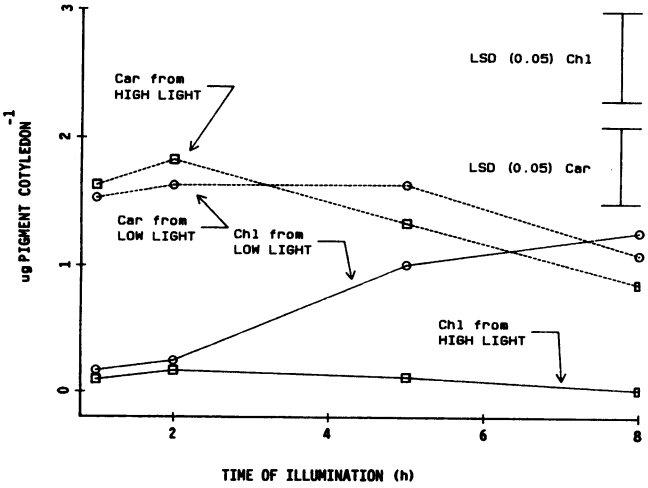


FIG. 5. Pigment levels in cotyledons treated with 5.0 $\mu$M AFM as a function of time and light intensity. Cotyledons were treated with 0.1% acetone (Controls) or 5 $\mu$M AFM, under green safelights, then incubated in a shaking flask in the dark for a 2 h uptake period. The treated tissue was washed in 1 mM $CaCl_2$, floated on untreated buffer, then placed under illumination of one of two intensities: 600 $\mu E/m^{-2}\cdot s^{-1}$; PAR (high light, □) or 150 $\mu E/m^{-2}\cdot s^{-1}$; PAR (low light, O) at time zero. Treatment sets of five cotyledons were sampled at the indicated time points and extracted for spectrophotometric pigment analysis. The levels of Chl (Chl ——) and carotenes (Car ——) were determined by the method of Lichtenthaler and Wellburn (12).

processes of interest. Following a suggestion offered by Itzhak Ohad of the Hebrew University, it was found that by exposing treated cotyledons to 1 min of low intensity illumination in 10 min cycles, it was possible to initiate significant greening in the cotyledons without inducing a measurable loss of membrane integrity. Tissue illuminated in this manner for 4 or 8 h in the presence of 5 $\mu$M AFM, then held in the dark for 20 h to allow detection of slow leakage, did not show efflux levels different from untreated controls. Therefore, we concluded that it was possible, by this means, to initiate pigment and plastid development in AFM treated cotyledons without damaging the plasmalemma. Treatments held under continuous light of the same intensity—25 $\mu E/m^{-2} \cdot s^{-1}$ (PAR)—for 8 h, did show small, but statistically significant increases in efflux relative to the controls; 4% leakage in 5 $\mu$M AFM *versus* 2% in controls. Apparently, as long as the period of intermittent light exposure was less than of a critical duration, the endogenous radical quenchers could prevent irreversible membrane damage from occurring.

This technique made it possible to begin to determine the relative effect of the greening process on AFM activity. Towards this end, etiolated cucumber cotyledons were floated on untreated buffer and exposed to one of three different treatment regimes, conducted in parallel as indicated in the diagram in Figure 6. The important experimental variable among the treatments was the condition of herbicide loading. In the first treatment set (S1), cotyledons were initially greened under intermittent lights for 4 h and then loaded with AFM in the dark. The second set (S2) was loaded with herbicide during a greening period identical to that in S1; the third set (S3) was also loaded with herbicide in the dark, but was never exposed to intermittent light prior to the transfer to continuous, high intensity illumination to which all treatments were exposed, at the same time, following the 4 h herbicide uptake period. At lower AFM concentrations, cotyledons greened in the presence of the herbicide (S2) were much more severely injured than those which, despite having received an identical period of intermittent illumination, were loaded with herbicide in the dark (S1). This differential tended to damp out at higher AFM concentrations; but in all cases, tissue pregreened under intermittent lighting prior to the transfer to continuous, high intensity illumination, sustained much greater injury than etiolated tissue (Fig. 7). Furthermore, it is important to note that the 5 $\mu$M treatments, loaded with herbicide under intermittent lighting, showed levels of response after the 3 h high light exposure equal to the levels of efflux recorded for the same rates after 16 h of low intensity (150 $\mu E\ m^{-2}\ s^{-1}$:PAR) illumination (*cf.* Figs. 2 and 7). The similarity in the levels of activity under these two different experimental conditions shows that the photoinhibitory effect of the continuous high light regime on greening, and not some deficiency of the emission spectrum in the high intensity illumination source, was responsible for the relative reductions in activity that were observed in the initial 16 h duration experiments (Fig. 1). Note further, that at the 5 $\mu$M rate, the level of efflux from S2 treatments after 3 h exposure to high intensity light, is 2 to 3 times greater than the efflux from initially etiolated tissue exposed to the same illumination for 16 h.

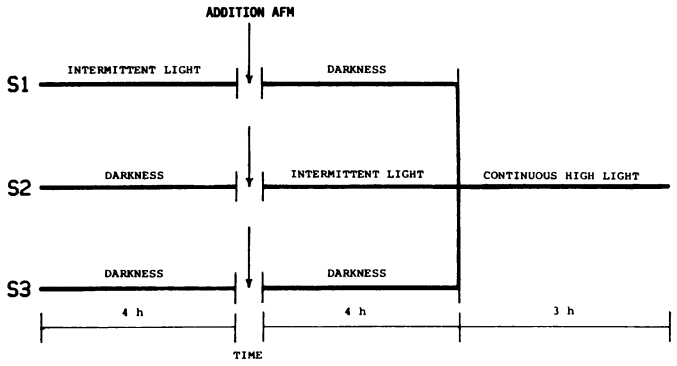


FIG. 6. Outline of the pretreatment regimes used to determine the relation of herbicidal efficacy to greening in AFM treated cotyledons. Cotyledons were floated on untreated buffer and at time zero, were either greened under 4 h of intermittent illumination, 1 min of light in 10 min cycles, (S1) or held in darkness for the same period of time (S2 and S3). AFM was then added to all treatments in each of the incubation regimes. The pregreened cotyledons (S1) were transferred to the dark and one set of previously dark incubated treatments (S2) were exposed to intermittent light during the herbicide uptake period. The important differentiating factor was the condition of herbicide uptake: S1 loaded in the dark after greening, S2 during greening, and S3 was not exposed to light either before or during AFM uptake. Following the herbicide uptake period, all cotyledons were removed from the treated media, rinsed in 1 mM $CaCl_2$, then were simultaneously placed under a continuous, high intensity illumination source (600 $\mu E/m^{-2} \cdot s^{-1}$; PAR) for 3 h to induce light activated membrane disruption.

Comparison of pigment extracts made from the various preincubation regimes described in Figure 6, taken prior to the exposure to continuous light, indicated that Chl levels in treatments loaded with herbicide after the intermittent light exposure were actually higher than in treatments loaded during greening, 7.6 *versus* 2.6 $\mu$g Chl cotyledon$^{-1}$ (Table I). This again seemed to suggest that interactions between the herbicide and the greening process, are more important in optimizing activity than the gross amount of photosenitizing pigments. The pigment measurements of Table I also show that 5 $\mu$M AFM significantly inhibited the accumulation of green pigments under conditions in which no measurable loss of plasmalemma integrity was observed, that is, the S2 loading regime. This suggests either that AFM specifically inhibited some step in Chl synthesis or that it was able to initiate photooxidation of green pigments without significantly disturbing the plasmalemma.

Stimulation of activity after intermittent lighting could also have been the result of greater herbicide uptake under this condition than in cotyledons incubated in the dark. Experiments were run to examine the effect of greening on activity, independent of possible differentials in the loading of AFM. Etiolated cotyledons were loaded with AFM in the dark and then removed from the herbicide treatments so that subsequent uptake could

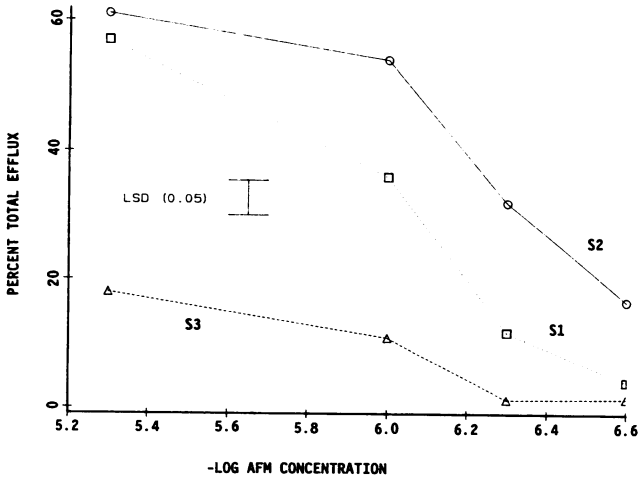


FIG. 7. Influence of greening on AFM activity. The conditions of AFM uptake prior to the exposure to continuous light were as described in the diagram in Figure 6: S1, loaded after greening (□···□); S2, loaded during greening (○——○); S3, loaded without greening (△——△). The extent of light activated membrane disruption was determined by sampling the treatment media for effluxed 3-O-methyl-[U-$^{14}$C]glucose.

Table I. *Pigment Accumulation in Cotyledons Treated with AFM after, during, or before Intermittent Illumination*

Measurements were made of the pigment levels in cotyledons from the various pretreatment regimes described in Figure 6, the pigment extraction was initiated after the 4 h herbicide loading period, but before the exposure to continuous, high intensity light. Cotyledons were floated on untreated buffer and at time zero, were either greened under 4 h of intermittent illumination, 1 min of light in 10 min cycles (S1), or held in darkness for the same period of time (S2 and S3). AFM was then added to all treatments in each of the incubation regimes. The pregreened cotyledons (S1) were transferred to the dark and one set of previously dark incubated treatments (S2) were exposed to intermittent light during the herbicide uptake period. The important differentiating factor was the condition of herbicide uptake: S1 loaded in the dark after greening, S2 during greening, and S3 was not exposed to light either before or during AFM uptake. The tissue was then extracted for pigment analysis according to the method of Lichtenthaler and Wellburn (12).

| Treatment Regime | AFM | Chl | Carotene |
|---|---|---|---|
| | μM | μg pigment cotyledon$^{-1}$ | |
| AFM loaded after greening S1 | 0 | 7.0 | 2.7 |
| | 5 | 7.6 | 2.7 |
| AFM loaded during greening S2 | 0 | 4.4 | 1.8 |
| | 5 | 2.6 | 1.9 |
| No greening S3 | 0 | 0.6 | 1.8 |
| | 5 | 0.6 | 1.8 |

LSD (0.05) Carotene = 0.3 Chl = 0.9

not occur by placing in covered Petri dishes without media, thus preventing back diffusion from becoming a significant factor. The cotyledons were then held in the dark or illuminated intermittently, as described in Figure 6, then exposed to continuous high intensity light. In these experiments, the period of herbicide uptake and the duration of exposure to continuous illumination were less than in the experiments of Figure 6 and so actual levels of efflux were also reduced. But the proportional differences between the S2 and S3 treatments in Figure 7, and the dark-held and intermittently illuminated treatments described above, were equivalent showing that the stimulation in activity affected by pregreening is not due to greater herbicidal uptake (Table II).

Duke *et al.* (4) had observed that mitochondrial inhibitors reduced the herbicidal activity of acifluorfen in etiolated cucumber cotyledon tissue. The mitochondrial inhibitors would have been expected to reduce ATP levels which would lead to an inhibition of Chl synthesis (23, 26). This suggested that metabolic inhibitors which affect greening, might be used to mimic the effect of high light intensities on etiolated tissue, to modulate AFM activity. Chloramphenicol can be used to inhibit the formation of chloroplast encoded proteins required for the assembly of the reaction centers, but not the LHCP which is synthesized in the cytoplasm under nuclear control (1, 3, 13). Integration of antennae Chl with LHCP in the lamellae allows significant greening to take place in the presence of chloramphenicol (8). Cycloheximide, a potent inhibitor of protein synthesis within the cytoplasm, at sufficiently high concentrations effectively prevents any greening from taking place (17). These antibiotic treatments had little effect on carotene levels in cucumber cotyledons after an initial 4 h exposure to intermittent illumination, but the high rate of cycloheximide severely inhibited Chl synthesis (Table III). When control treatments were pregreened in this manner, Chl synthesis was able to continue after transfer to sustained illumination intensities of 600 $\mu E/m^{-2} \cdot s^{-1}$ (PAR) which previously had proven inhibitory to Chl accumulation in etiolated tissue when transferred directly from the dark (Table III) *versus* Fig. 3). Following the period of continuous illumination, there was little variation in carotene levels among the protein inhibitor

Table II. *Herbicide Efficacy in Etiolated and Greening Cotyledons under Conditions of Fixed or Variable AFM Uptake*

Etiolated cotyledons were floated, on media treated with 5 μM AFM, in the dark for 4 h. At the end of this uptake period, all media was removed from all treatments and cotyledons were incubated in covered Petri dishes either in the dark, or under intermittent illumination (1 min of light in 10 min cycles) for 4 h. All treatments were then simultaneously placed under a continuous, high intensity illumination source (600 $\mu E/m^{-2} \cdot s^{-1}$; PAR) for 2 h to induce light activated membrane disruption. Under these conditions, herbicide uptake was fixed at the same level in etiolated and greening treatments by the removal of the treatment media which prevented subsequent AFM loading and/or back diffusion. Following the exposure to continuous light, herbicidal activity was determined by refloating and shaking the cotyledons on untreated buffer for 1 h in the dark before the wash media was sampled for effluxed 3-O-methyl-[U-$^{14}$C]glucose. Efflux is expressed as a percent of the total uptake of radioactivity. Values from the 5 μM S2 and S3 treatments of Figure 7, where herbicide uptake might have varied during the pregreening treatments, have been included for the sake of comparison.

| Treatment | Activity of 5 μM AFM |
|---|---|
| | % total potential efflux |
| Fixed herbicide uptake | |
| Pregreened | 24 |
| Etiolated | 7 |
| Variable herbicide uptake | |
| S2 | 61 |
| S3 | 18 |

LSD (0.05) for 3.4% for fixed uptake, 5.4% for variable uptake

Table III. *Pigment Accumulation in Cotyledons Treated with Protein Synthesis Inhibitors*

Etiolated cotyledons were floated on media treated with the specified inhibitors, in the dark for 1.5 h. All treatments were then exposed to intermittent illumination (1 min of light in 10 min cycles) for 4 h, after which replicates from each treatment were extracted for pigment analysis according to the method of Lichtenthaler and Wellburn (12). The remaining replicates from each treatment were then illuminated under continuous, high intensity light for 2 h prior to extraction.

| Treatment | After 4 h Intermittent Illumination | | After Additional 2 h Continuous High Intensity Illumination | |
|---|---|---|---|---|
| | Chl | Carotene | Chl | Carotene |
| | μg pigment cotyledon$^{-1}$ | | | |
| Control | 3.8 | 1.6 | 6.0 | 2.3 |
| 2.0 mM chloramphenicol | 3.7 | 1.5 | 3.7 | 1.8 |
| 1.0 μM cycloheximide | 2.7 | 1.6 | 3.2 | 2.0 |
| 10.0 μM cycloheximide | 0.8 | 1.7 | 0.4 | 1.7 |

LSD (0.05) Carotene = 0.02 Chl = 0.7

treatments and the overall pigment levels of 1 μM cycloheximide and 2 mM chloramphenicol treatments were not significantly different. When cotyledons were treated with herbicide in combination with the antibiotics, the highest rate of cycloheximide, which prevented greening, also proved to be the most effective in reducing AFM activity (Fig. 8). The chloramphenicol treatments did cause some slight reduction in the response to 0.5 μM AFM, but had no effect on the activity of 5 μM concentrations of the herbicide. Interestingly, the 1 μM cycloheximide and 2 mM chloramphenicol treatments, which had been shown to result in similar total pigment levels in nonherbicide treated cotyledons, differed significantly in their effect on AFM activity. Certainly

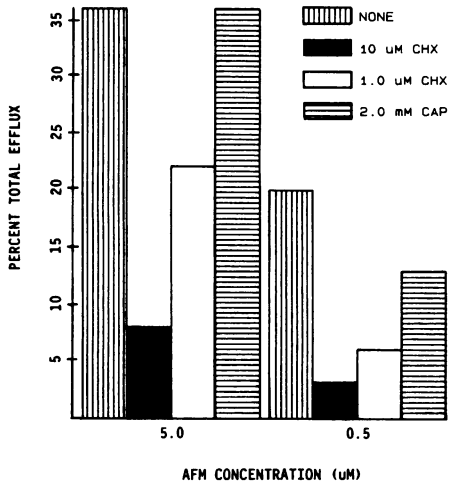


FIG. 8. Suppression of AFM activity by protein synthesis inhibitors. Cotyledons were pretreated by floating on media, with CHX, cycloheximide; or CAP, chloramphenicol; for 1.5 h in the dark. AFM was then added to the media, and the treatments were then exposed to intermittent illumination (1 min of light in 10 min cycles) for a period of 4 h. All treatments were then simultaneously placed under a high intensity illumination source (600 $\mu E/m^{-2}\cdot s^{-1}$; PAR) for 2 h. The extent of light activated membrane disruption was determined by sampling the treatment media for effluxed 3-O-methyl-[U-$^{14}$C]glucose.

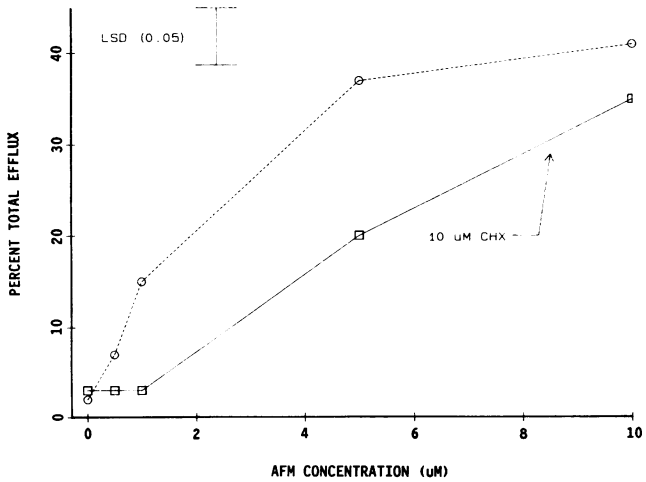


FIG. 9. Effect of cycloheximide on the AFM dose response. Etiolated cotyledons were treated simultaneously with 10 $\mu$M cycloheximide and AFM (□——□), or AFM alone (○——○), and held for 8 h in darkness. All treatments were then placed under a continuous, high intensity illumination source (600 $\mu E/m^{-2}\cdot s^{-1}$; PAR) for 16 h. The extent of light activated membrane disruption was determined by sampling the treatment media for effluxed 3-O-methyl-[U-$^{14}$C]glucose.

in an *in vivo* system as complex as the cotyledons, the protein synthesis inhibitors could have secondary effects on a number of processes not related to plastidic greening which could also affect AFM activity. For example, reduction in herbicidal activity would be expected if cycloheximide somehow reduced AFM uptake into treated cotyledons or altered the intercellular compartmentalization of the herbicide. The inhibitor effects do not conclusively link AFM activity with greening, but are merely consistent with the results from the various lighting regime experiments. Of greater significance were experiments conducted with etiolated cotyledons dark incubated for an extended period (8 h) with 10 $\mu$M treatments of cycloheximide and various rates of AFM, prior to exposure to continuous high intensity light. Chl synthesis in the cotyledons pretreated with cycloheximide was effectively blocked, there was no detectable Chl in tissue treated with 10 $\mu$M cycloheximide alone, following the continuous illumination period. Yet even in cotyledons pretreated in this manner, AFM was able to initiate light dependent membrane disruption, although significant levels of efflux were only observed at relatively high rates, 5 $\mu$M and above (Fig. 9). This shows that even in plant tissues where Chl synthesis is severely inhibited, but *in situ* carotenes are retained, AFM can be activated.

Apparently, processes associated with greening in etiolated cotyledons, that are inhibited by cycloheximide or high intensity illumination, are not absolutely essential for the herbicidal activation of AFM, but do significantly influence the level of that activity. A direct interaction between a Chl/protein complex and the DPE herbicides could account for a mechanism, other than photosynthetic electron transport, by which Chl could play a role in the activation of these herbicides. There seem to be a number of possible explanations for the greater herbicidal response observed in systems greened in the presence of AFM. If the herbicide molecule is directly involved with the transfer to electrons from pigments to oxygen, then a more highly optimized association may occur between the herbicide and the photosensitizing site in a newly forming pigment-protein complex than might take place between the herbicide and a preformed complex. Alternatively, the DPE herbicides may disrupt the proper formation or integration of pigment complexes resulting in the incorporation of dysfunctional units which photoreduce oxygen rather than carrying out proper electron transfers (18). Yet another possibility is suggested by the inhibition of Chl accumulation, by AFM, under intermittent illumination indicated in Table I. Prevention of the complete processing of pigment intermediates and their subsequent incorporation into the lamellae might lead to the accumulation of photodynamic Chl precursors (2). These natural photosensitizers could then make a significant additive contribution to AFM activity.

Although many are effective as preemergence treatments, DPE herbicides are most typically applied to weeds, in commercial usage, only after the plants are emerged from the soil. However, these herbicides are most effective in very young seedlings and older more mature plants are controlled only with difficulty (11, 14, 16). Therefore, there does seem to be a potential for these compounds to interact with Chl synthesis in the young, actively growing seedlings which are the recommended target of field treatments.

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