Plant Physiol. (1988) 86, 619–622
0032-0889/88/86/0619/04/$01.00/0

Studies on the Mode of Action of Acifluorfen-Methyl in Nonchlorophyllous Soybean Cells[1]

ACCUMULATION OF TETRAPYRROLES

Received for publication June 23, 1987 and in revised form September 14, 1987

M. MATRINGE* AND R. SCALLA
Laboratoire des Herbicides, Institut National de la Recherche Agronomique, BV 1540, 21034 Dijon, France

ABSTRACT

Phytotoxic effects of the herbicide acifluorfen-methyl on nonchlorophyllous soybean cells were estimated by ^{86}Rb leakage. An action spectrum study showed maximum injury at 350 to 450 nanometers, with lesser activity between 450 and 700 nanometers. Cells treated in the dark with acifluorfen-methyl accumulated fluorescent pigments with the spectral characteristics of protoporphyrin IX. The action spectrum of acifluorfen-methyl matched the absorption spectrum of this tetrapyrrole, and the extent of cellular damage in the light was related to the degree of fluorescent pigment accumulation. We propose that the phytotoxicity of diphenyl ether herbicides could be explained by their ability to cause abnormal accumulations of tetrapyrroles, which in turn induce lethal photooxidative reactions.

Diphenyl ether herbicides have an absolute requirement for light for their action (17, 19). In the presence of O_2, they induce photooxidative reactions leading to degradation of fatty acids, as shown by the formation of ethane and thiobarbituric acid-reacting materials (2, 13, 15, 19). The resulting membrane disruptions lead to cellular lysis (15, 19).

In spite of numerous studies, the exact nature of the light-activated mechanism remains unknown. AFM[2] itself does not absorb visible light, but there is evidence that it could act as a photosensitizer *in vitro* (20, 21), at least under UV irradiation (21). However, its exact participation in photodynamic reactions *in vivo* remains obscure, and, for instance, the role of DPE free radicals is uncertain (7). Thus, cellular photoreceptors are apparently required for the herbicidal action of DPEs. It has been repeatedly demonstrated that, contrary to green or etiolated seedlings which are sensitive, plants deprived of their chloroplastic pigments are resistant (3, 5, 15, 17, 19). More precisely, studies involving chloroplastic mutants have led to the assumption that the phytotoxic process is mediated by carotenoid pigments (17).

Though generally accepted, this theory meets at least two serious difficulties. First, in chlorophyllous seedlings (15), as well as in green algae (8, 14), DPE toxicity can be induced by red light *i.e.* outside the absorption range of carotenoids. Second, it has been found that, contrary to green tissues, nonchlorophyllous soybean cells remain sensitive after being deprived of their carotenoids by norflurazon treatment (16). These results thus raise doubts about the role generally ascribed to carotenoid pigments.

Owing to these discrepancies, we have examined the action spectrum for the toxicity of a DPE herbicide, AFM, on nonchlorophyllous soybean cells. Our results lead us to propose a new scheme for the mode of action of DPE herbicides.

MATERIALS AND METHODS

Cell Culture. Nonchlorophyllous soybean cells were grown in the light (15 μE m^{-2} s^{-1}), in a liquid medium as described earlier (16). Before the experiments, 3 d-old subcultures were diluted to 30 mg fresh weight ml^{-1}. All chemical treatments were done in the dark, during a 14 h incubation period.

Action Spectrum. The light source was a slide projector, and action spectra were obtained using Balzers broadband interference filters, type Filtraflex-K. For all wavelengths, cells were submitted to a light intensity of 300 μE m^{-2} s^{-1}. For white light irradiation, we used incandescent lamps (MAZDA PAR, cool beam, 120 W) giving 300 μE m^{-2} s^{-1}.

Membrane Integrity. Loss of membrane integrity was estimated from the amount of ^{86}Rb released by cells into the culture medium, according to Orr and Hess (19). Cells were incubated for 14 h in the dark with ^{86}Rb Cl ($1–1.5 \times 10^5$ cpm ml^{-1} cell suspension), in the presence or absence of AFM and other chemicals. Just before light exposure (650 μE m^{-2} s^{-1}), cells were centrifuged twice at 10,000*g* for 10 min, and resuspended in a culture medium containing the same chemicals as before, but without ^{86}Rb. At intervals, 1 ml aliquots of cell suspensions were centrifuged, and the radioactivity of 500 μl of supernatant was determined by liquid scintillation spectrometry. Zero time values were subtracted from countings in all experiments.

Protective Effect of 4,6-Dioxoheptanoic Acid and Antimycin A. DA was added twice to the cell suspension (final concentration 1 mM) at the beginning of the 14 h dark pretreatment, and 1 h before light exposure (650 μE m^{-2} s^{-1}). Antimycin A (final concentration 2 μM) was given at the beginning of the dark pretreatment.

Extraction of Tetrapyrroles. This extraction was done according to Rebeiz *et al.* (22). Cell suspension (25 ml) was filtered and homogenized in 20 ml acetone/0,1 M NH_4OH (9/1, v/v). The resulting acetone extract was cleared of lipoproteins and cell debris by centrifugation at 30,000*g* for 10 min at 0°C. Carotenoids and fully esterified tetrapyrroles were removed from the aqueous acetone solution by extraction with hexane (22). The tetrapyrrole content of the hexane-extracted acetone was determined spec-

[1] Supported by a grant from Institut National de la Recherche Agronomique (AIP 4483).

[2] Abbreviations: AFM, acifluorfen-methyl; DPE, diphenyl ether; DA, 4,6-dioxoheptanoic acid; LS 82-556, ([*S*]3-*N*-[methylbenzyl]carbamoyl-5-propionyl-2,6-lutidine).

trofluometrically using a Jobin et Yvon 3D fluorimeter calibrated with a 1 to 8 $\times$ 10^{-7} M solution of protoporphrin IX.

Chemicals. Chemicals were dissolved in dimethyl sulfoxide or acetone. Final concentrations of solvents in the cell suspension were 1% (v/v) or less, and appropriate controls were run. Antimycin A, DA, and protoporphyrin IX were obtained from Sigma. AFM and LS 82-556 were gifts from Rhône-Poulenc Agrochimie, France.

Statistics. All experiments were repeated at least three times with not less than two replicates.

RESULTS

Action Spectrum of AFM. We have previously found that the sensitivity of nonchlorophyllous soybean cells to AFM is strictly light-dependent (16). Figure 1 shows that upon irradiation with light, cells treated with AFM released more ^{86}Rb than control cells, which indicated membrane damage. Maximum response occurred in the 350 to 450 nm region transmitted by a K 40 filter. Wavelengths between 450 and 700 nm also induced membrane disruption but were less efficient. Wavelengths transmitted by the K 70 filter did not induce significant damages, suggesting a drop in activity above 700 nm.

These results indicate that the chromophore(s) implicated in the toxic process strongly absorbs light in the blue region (400 nm) and has secondary zones of absorption at wavelengths from 450 to 700 nm. As already reported (16), we ruled out a particip-

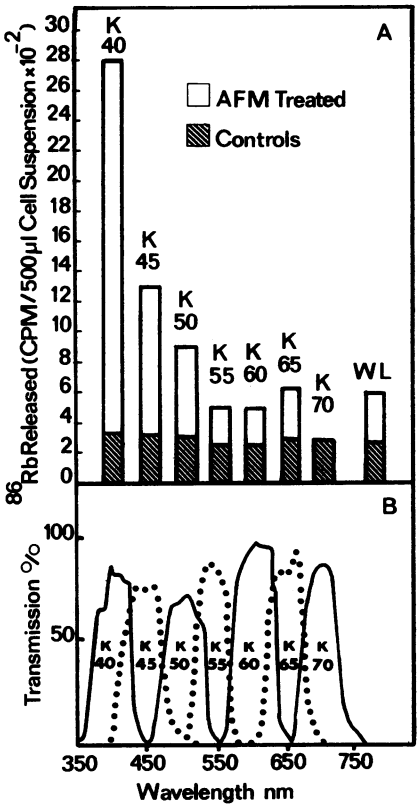

FIG. 1. A, Action spectrum of the DPE herbicide AFM. Nonchlorophyllous soybean cells were incubated with ^{86}RbCl and 10 μM AFM for 14 h in the dark. They were then exposed for 4 h light of the different wavelength bands. Light intensity was 300 μE m^{-2} s^{-1} for all treatments. Membrane disruption was estimated by the release of ^{86}Rb into the culture medium. This experiment was repeated five times in slightly different conditions. The figure shows the result of one representative experiment. WL, White light; (▨), Control (no AFM); (□), AFM treated. B, Transmission characteristics of the filters, recorded with a Shimadzu UV 240 spectrophotometer.

ipation by carotenoids with respect to our soybean cell cultures. Since these cells are devoid of Chl (16), a role for these pigments is also excluded. Among other possible candidates, tetrapyrroles have an absorption spectrum matching the DPE action spectrum, since they show a Soret peak in the blue region and secondary bands in the green, yellow, orange, and red regions of the spectrum (4). Therefore, we investigated the possible implication of tetrapyrroles as photoreceptors.

Protective Effect of 4,6-Dioxoheptanoic Acid. If the photoreceptors for the light-activated mechanism are tetrapyrroles, cells deprived of these molecules should lose their sensitivity to AFM. In order to suppress tetrapyrrole synthesis, we have used DA, an inhibitor of δ-aminolevulinic acid dehydratase (18). Figure 2 shows that a 14 h pretreatment in the dark with 1 mM DA markedly reduced cell sensitivity to AFM in the light. These results raised the possibility that tetrapyrroles could play a role in the light-activated phytotoxicity of AFM.

Accumulation of Tetrapyrroles in AFM-Treated Cells. When tetrapyrroles were measured in hexane-washed acetone extracts of control cells, tetrapyrrole concentration was below detection level. By contrast, extracts of cells treated with AFM (1 or 10 μM) for 14 h in the dark showed the fluorescence characteristics of protoporphyrins (23), with an excitation maximum at 405 nm and an emission maximum at 663 nm (Fig. 3). These spectra closely matched the fluorescence signals of a protoporphyrin IX standard (Fig. 3). The same result was found with the experimental herbicide LS 82-556 (Fig. 3C), which although structurally unrelated to DPE, exerts the same type of toxicity (15). When expressed as protoporphyrin IX equivalents, tetrapyrrole accumulation amounted to 40 to 50 and 60 to 80 nmol/g dry weight for cells treated with 1 and 10 μM AFM, respectively, and 16 to

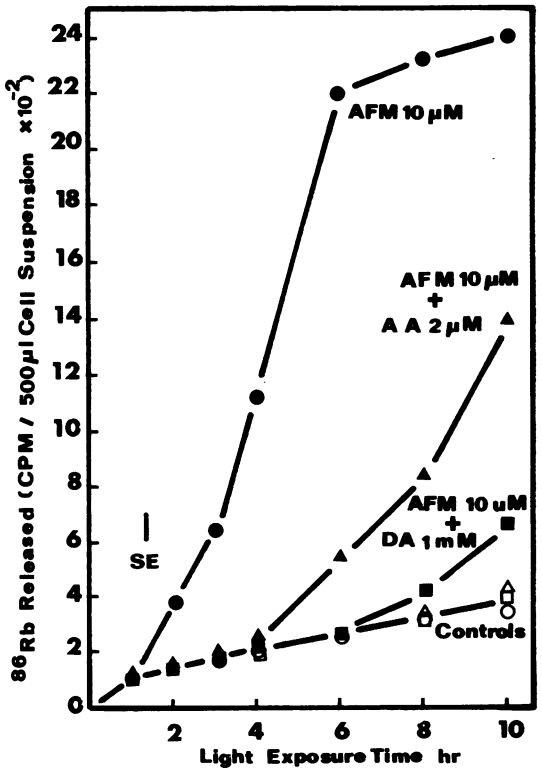

FIG. 2. Effect of DA, and antimycin A (AA) on the amount of ^{86}Rb released under white light (650 μE m^{-2} s^{-1}). Nonchlorophyllous soybean cells were treated for 14 h in the dark with 10 μM AFM (●), 10 μM AFM + 2 μM antimycin A (▲), or 10 μM AFM + 1 mM DA (■), and then put in the light (650 μE m^{-2} s^{-1}). Controls are represented by the corresponding open symbols. Vertical bars represent standard error.

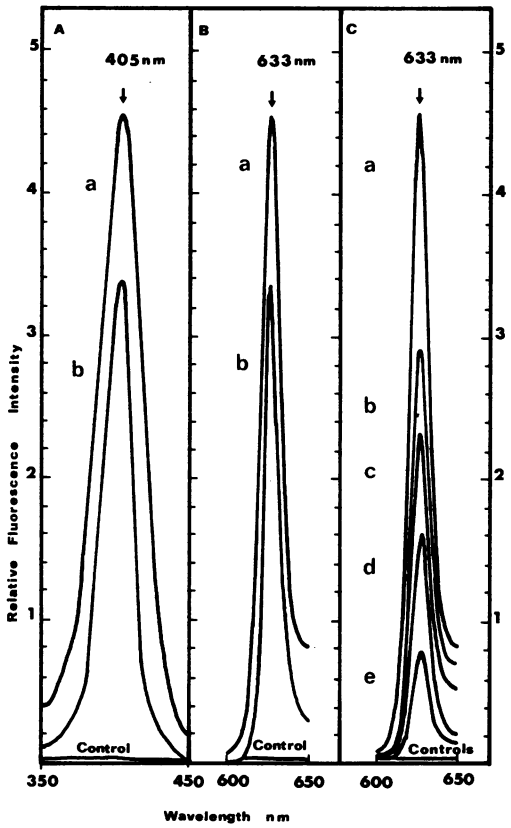

FIG. 3. Fluorescence excitation and emission spectra of acetone extracts of cells submitted to various treatments for 14 h in the dark. A, Excitation spectra recorded at 633 nm; B, emission spectra elicited at 405 nm: a, hexane-washed acetone extract of 25 ml of cell suspension treated with 10 μM AFM; b, hexane-washed acetone solution of protoporphyrin IX standard (6×10^{-7} M). C, Emission spectra elicited at 405 nm. Cells treated with 10 μM AFM (a), 1 μM AFM (b), 10 μM AFM + 2 μM antimycin A (c), 100 μM LS 82-556 (d), and 10 μM AFM + 1 mM DA (e). Concentrations of tetrapyrroles, estimated as protoporphyrin IX equivalents, were: 40 to 50 and 60 to 80 nmol/g dry weight for cells treated by 1 and 10 μM AFM, respectively; 28 to 35 nmol/g dry weight for AFM + antimycin A-treated cells; 16 to 23 nmol/g dry weight for LS 82-556-treated cells; and 10 to 14 nmol/g dry weight for AFM + DA-treated cells.

23 nmol/g dry weight for cells treated with 100 μM LS 82-556. This accumulation was markedly reduced if AFM treatment was done in the presence of 1 mM DA (Fig. 3C). The accumulation of protoporphyrins was confirmed by the absorption spectra of hexane-washed acetone extracts, which showed a Soret peak near 400 nm and secondary absorption bands in the green, yellow, orange, and red regions of the spectrum (Fig. 4). These absorption spectra matched that of protoporphyrin IX (Fig. 4).

Protective Effect of Antimycin A. We have previously shown that antimycin A protects soybean cells against AFM toxicity (16). This protection suggested a role of the mitochondrion electron transfer chain, but the exact nature of this participation remained unclear. This effect of antimycin A was confirmed in the present experiments (Fig. 2), and we found that antimycin A reduced the accumulation of tetrapyrroles in AFM-treated cells (Fig. 3C).

DISCUSSION

The nature of the cellular photoreceptors mediating DPE-induced photooxidations is still controversial. In spite of the evidence accumulated in favor of carotenoids, the exact role of these pigments remains unclear, and our previous work (16) has led us to reappraise this point.

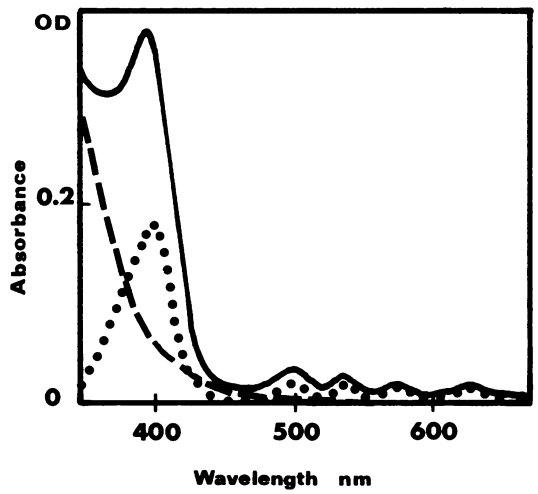

FIG. 4. Absorption spectra of the hexane-washed acetone extracts of control cells (– – –), of cells treated with 10 μM AFM (——), and of a 4×10^{-7} M solution of protoporphyrin IX in hexane-washed acetone (●●●).

Measurement of action spectra should provide a logical approach toward identificaton of DPE photoreceptors. Unfortunately, conclusions can be obscured by the presence of numerous cellular pigments which interfere with these spectra, especially in green tissues. To circumvent, at least partially, this difficulty, we used nonchlorophyllous cell cultures. These cells have proved to be sensitive to micromolar concentrations of AFM in a strictly light-dependent manner (16), thus acting like chlorophyllous tissues (2, 19).

We have found in this study that AFM exerts most of its phytotoxicity in the blue region of the spectrum, with lesser effects from 450 to 700 nm. This result contrasts with the conclusions of Vanstone and Stobbe (25), who found that toxicity of oxyfluorfen on buckwheat leaf discs occurs mainly between 515 and 615 nm, with little or no activity in the blue or red wavelengths. The action spectrum presented here also differs from that established on green algae by Esminger and Hess (8). These authors showed that toxicity of AFM was induced in the blue and red regions, suggesting a participation by Chl and carotenoids. Obviously, the absence of Chl from our soybean cells can at least partly explain the lack of agreement between our results and those of other authors.

The shape of the action spectrum reported here matches with the absorption spectrum of protoporphyrin IX (Fig. 4). This suggests that tetrapyrroles or more precisely protoporphyrins, could mediate the absorption of light energy required for the herbicidal action of AFM, and this view is supported by several lines of evidence:

1. Treatment of cells in the dark with AFM induced an accumulation of pigment(s) with the fluorescence and absorption characteristics of protoporphyrin IX (Figs. 3 and 4). Moreover, this accumulation was found at micromolar concentrations of AFM, and the level of this accumulation increased with the concentration of AFM (Fig. 3).

2. DA, an inhibitor of tetrapyrrole biosynthesis, reduced the accumulation of the fluorescent pigment (Fig. 3C), and simultaneously lessened AFM toxicity (Fig. 2).

3. Antimycin A, which protects soybean cells against AFM toxicity (Fig. 2), also reduced the AFM-induced accumulation of tetrapyrroles (Fig. 3C).

4. LS 82-556, which is chemically unrelated to DPE, nevertheless exerts exactly the same morphological, cytological, and biochemical effects in a strictly light-dependent manner (15, 16). This compound also induced an accumulation of fluorescent pigment(s) (Fig. 3C). LS was previously found less potent than AFM on soybean cells (16). Accordingly, its effect on pigment accumulation was found at a higher concentration than AFM (Fig. 3C).

Taken together, our experimental data lead us to propose that the light-activated phytotoxicity of AFM can be explained by its ability to induce an abnormal accumulation of tetrapyrroles. These pigments are known to be powerful, light-dependent singlet oxygen generators (12). In turn, singlet O_2 would be responsible for the peroxidative degradation of cellular membrane-fatty acids which is seen after DPE treatment. Accumulation of tetrapyrroles in plants treated by δ-aminolevulinic acid and 2,2′-dipyridyl, has been shown to result in a similar light-dependent toxicity (24).

This hypothesis is strengthened by the fact that LS 82-556, which induces exactly the same biochemical symptoms as DPE, also evokes tetrapyrrole accumulation. Thus, this effect may not be particular to AFM, but probably the general mode of action of DPE-type herbicides.

Figure 5 is based on the data accumulated on DPE activity. It suggests how DPE herbicides induce fatty acid radical formation without participating directly in redox or radical reactions (7, 20). These reactions have also proved elusive to detect in the case of LS 82-556, in spite of repeated attempts (P. Meallier, unpublished results). The scheme might explain the controversial role of chloroplasts (2, 6, 9, 10, 15, 19) in the toxic process, as well as the possible role of mitochondria (5, 16). These organelles are sources of δ-aminolevulinic acid, and they are the sites of the final steps of heme and Chl synthesis (1, 11). Moreover, our scheme could explain the failures to demonstrate DPE-induced peroxidative destruction in isolated thylakoid preparations (6, 9), which lack soluble precursors and thus are probably unable to perform any significant tetrapyrrole synthesis.

Finally, this theory leads to a seeming paradox. Although the phytotoxicity of DPEs requires light, these herbicides could induce an accumulation of tetrapyrroles even in the dark (Fig. 3). Their light requirement could concern, in fact, only the second part of the toxic process they induce, *i.e.* the photooxidative reactions. On the other hand, it is known that while producing singlet oxygen, tetrapyrroles disappear, probably as a consequence of photodestruction (24). We have noted such a disapperance of the tetrapyrroles accumulated during a dark pretreatment in our system, during the first hours of light exposure (650 $\mu E\ m^{-2}\ s^{-1}$, results not shown). This phenomenon could make it difficult to demonstrate tetrapyrrole formation in the light.

However, more detailed studies appear necessary to clarify these ideas, and to define precisely the mechanism by which DPEs induce protoporphyrin accumulation.

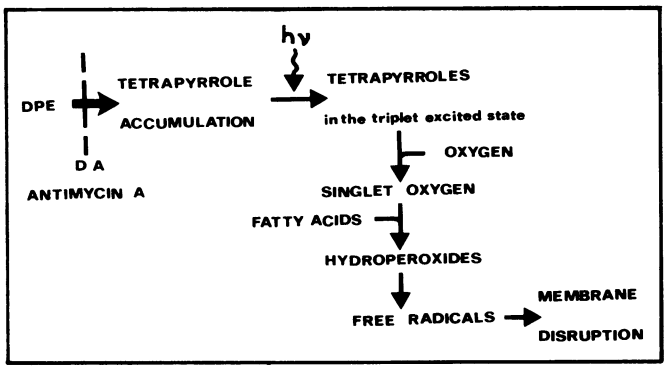

FIG. 5. Proposed scheme of the light-activated mechanism of action of DPEs on nonchlorophyllous soybean cells. (– – –), Protective effects of DA and antimycin A.

Acknowledgments—The authors thank Rhône-Poulenc Agrochimie (France) for supplying AFM and LS 82-556. The technical assistance of Denis Clair is gratefully acknowledged. The authors are indebted to Colin J. Straker for correction of the English manuscript.

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