Recent Advances in the Mode of Action of Diphenyl Ethers and Related Herbicides

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Z. Naturforsch. **45c,** 503–511 (1990); received November 6, 1989

Diphenyl Ethers, Herbicides, Oxadiazon, Peroxidizing Herbicides, Porphyrin, Chlorophyll Synthesis, Cytochrome Synthesis

Several hypotheses have been proposed to explain the light-dependent phytotoxicity of diphenyl ethers and related herbicides: inhibition of photosynthesis; activation of herbicides by light, by the photosynthetic electron transfer chain or by excited forms of carotenoids; and interaction with the biosynthesis of tetrapyrrole pigments. It is shown that the most likely mode of action consists in inhibiting the enzyme protoporphyrinogen oxidase. As a consequence, protoporphyrinogen is oxidized non-enzymatically to protoporphyrin IX. The latter molecule is a powerful photosensitizer, able to generate singlet oxygen in the light and thus to induce peroxidative destruction of membrane lipids.

Introduction

Although various substituted nitrodiphenyl ethers (DPEs) exert a number of modes of action [1], acifluorfen-methyl (AFM) and molecules possessing the same substituent pattern induce characteristic light-dependent phytotoxic damages. In addition, other molecules from unrelated chemical families apparently have a similar mode of action (Fig. 1).

It has long been realized that light is required for the phytotoxicity of DPEs [2]. That requirement can be easily demonstrated by observing the growth of treated plants (Fig. 2). When no or little effect can be observed in the dark, at least for a few days, growth is slowed or arrested upon exposure to the light. It is also possible to allow plants to absorb the herbicide in the dark, before starting the phytotoxic process by exposure to the light. In these conditions, external symptoms can be observed after a few hours of illumination, and Orr and Hess [3], working with cucumber cotyledons, have even observed physiological disturbances after only 15 min light. Similar conclusions have been reached concerning the pyridine derivative LS 82-556 [4], oxadiazon [5], M&B 39279 [6], and S-23142 [7].

Observations with the electron microscope show that the wilting and necrosis phenomena induced by DPE-type herbicides are caused by a generalized disruption of cell membranes [4, 6, 8, 32]. The ensuing cell lysis can be detected by measuring various parameters: loss of water, efflux of electrolytes or radioactive rubidium [3, 4, 9]. In the case

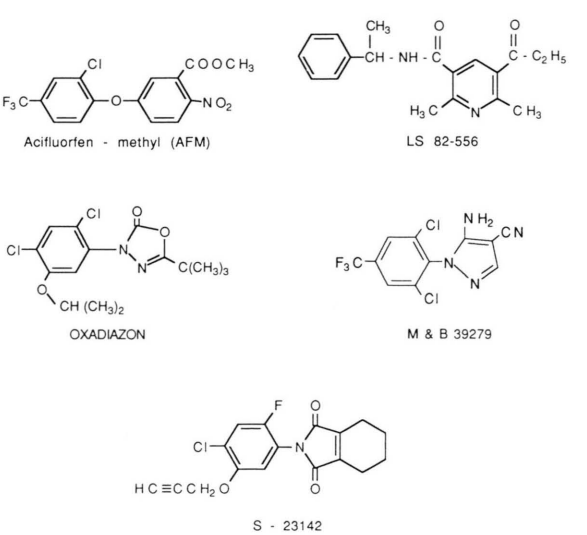

Fig. 1.

Abbreviations: ALA, δ-aminolevulinic acid; AF(M), acifluorfen(-methyl); DPEs, diphenyl ethers; Mg-Proto, Mg protoporphyrin IX; Proto IX, protoporphyrin IX.

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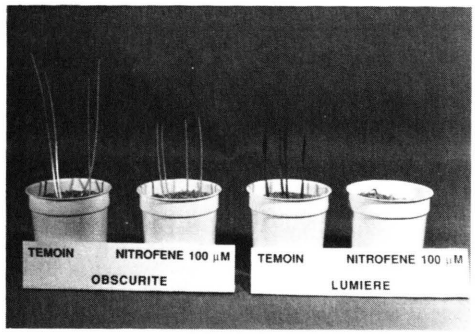

Fig. 2. Light-dependent phytotoxicity of the diphenyl ether nitrofen. Wheat seeds were sown in sand watered with nutrient solution with or without 100 µM nitrofen. The plant were photographed 7 days later.

of cell cultures, the efflux of a pre-loaded dye can also be measured [10].

Disorganization of cell membranes has been traced back to peroxidative destruction of lipids. Upon the formation of hydroperoxide derivatives, unsaturated fatty acids are degraded to alcanes and aldehydes, which have been detected in plant tissues treated with DPEs [11], with the pyridine derivative LS 82-556 [4], and with the N-phenylimide S-23142 [7]. Appearance of these degradation products can be observed before the onset of visible symptomes [4].

These observations have led to a preliminary hypothesis for the mechanism of action of DPE-type herbicides. That hypothesis can be summarized as follows: (1) Light is required for the phytotoxicity, and that implies the participation of a photoreceptor. (2) Since a number of DPE-type herbicides do not absorb visible light, the photoreceptor must be a cellular molecule. (3) It is thus conceivable that the herbicides are activated in some way by the photoreceptor, which can possibly operate in a more complex light-requiring cellular process. The hypothetical activated herbicides could then react with unsaturated fatty acids, giving rise to fatty acid radicals prone to be attacked by oxygen to produce unstable hydroperoxides. (4) Peroxidative breakdown of lipids and other cellular constituents [12] result in cell lysis.

In this scheme, points (1), (2) and (4) are indisputable. However, though a number of experiments have demonstrated the role of light, they have given contradictory clues to the nature of the photoreceptor. Moreover, the actual role of activated forms of herbicides has also been a matter of debate.

Interaction of diphenyl ethers and related herbicides with photosynthesis

Concerning the role of light, one of the first possibilities which has been explored is that of an interference of DPE-type herbicides with photosynthesis. Indeed, the biochemical symptoms evoked by these herbicides are very reminiscent of those of inhibitors of photosystem II, which induce lipid peroxidations mediated by the transfer of energy from excited chlorophylls to fatty acids or to ground state oxygen [13]. Moreover, the bipyridylium acceptors of photosystem I electrons induce similar effects by another mechanism, *i.e.* the formation of superoxide ions [13].

Various studies have shown that nitro-DPEs can inhibit the photosynthetic electron transfer in the plastoquinone-cytochrome *f* region [14], and that they can bind to the chloroplastic coupling factor [15]. It has been generally found, however, that their I_{50} values are in excess of 10^{-5} M [16], so that inhibition of photosynthesis cannot be a primary mode of action of DPEs. Moreover, Tissut *et al.* [17] have reached a similar conclusion concerning LS 82-556, which does not inhibit nor uncouple photosynthetic electron transfer in isolated chloroplasts. That molecule is not an electron acceptor, and does not inhibit the CO_2-dependent O_2 evolution of intact chloroplasts.

In contrast to these *in vitro* studies, use of DCMU or atrazine to probe the role of photosynthesis in the *in vivo* phytotoxicity of DPEs led to conflicting results. According to Duke *et al.* [18, 19], PS II inhibitors do not protect cucumber cotyledon discs against AF. By contrast, it was observed by Kunert and Böger [20] that diuron protects *Scenedesmus* cells against oxyfluorfen, and by Matringe *et al.* [4] that the same PS II inhibitor also protects cucumber seedlings against AFM or LS 82-556. These seemingly conflicting results can be reconciled by the observation that PS II inhibitors antagonize the effects of DPEs in chlorophyllous, autotrophic tissues (as shown for example in ref. [4]), when they do not afford any protection if the same green tissues are floated on a sucrose solution, and thus have a mixotrophic metabolism

[18, 19]. That led Nurit *et al.* [21] to suggest that an organic precursor needed for the photo-dependent toxic action of peroxidizing herbicides is provided by photosynthesis when the tissues are floated on water, and by respiration when the medium contains sucrose. That photosynthesis *by itself* is not required for the phytotoxicity of DPEs is clearly demonstrated by the marked light-dependent susceptibility of non-chlorophyllous cell cultures to AFM and LS 82-556 [10]. Indeed, these non-chlorophyllous cells, as well as chlorophyllous tissues incubated with sucrose, are protected by the respiration inhibitor antimycin A [10, 18], in agreement with the suggestion of Nurit *et al.* [21].

In addition to their possible inhibiting photosynthetic electron flow, it has been proposed that nitro-DPEs can accept electrons from PS II. Experiments done by Gilham and Dodge [22, 23] with pea leaf discs have shown that light-dependent herbicide injury was reduced by pre-incubation with the electron-transport inhibitor monuron. Ferredoxin-dependent $NADP^+$ reduction was repressed by DPEs in illuminated chloroplasts. Further experiment led the authors to propose that nitro-DPEs can accept electrons from reduced ferredoxin and are converted to activated species by that reaction.

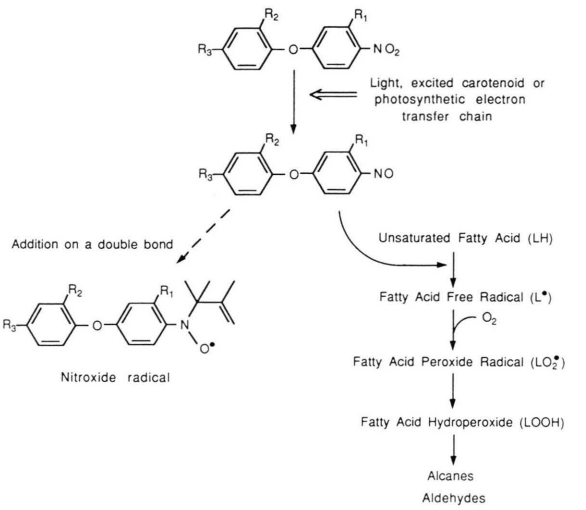

Fig. 3. Hypothetical mechanisms of action involving radical or reduced forms of a diphenyl ether herbicide. The reaction leading to a nitroxide radical is from Draper and Casida [24].

Models involving activated forms of diphenyl ether herbicides

Indeed, studies on the redox chemistry of nitro-DPEs indicated that, in artificial systems, these molecules can be reduced to nitroso derivatives apt to bind to unsaturated lipids (Fig. 3) and to initiate radical-mediated peroxidations [24]. In the same context, Lambert *et al.* [25] were able to detect radicals by ESR technique in chloroplast preparations illuminated in the presence of DPEs. Activity of the photosynthetic electron transport was required for the appearance of ESR signal.

These studies raise the possibility that reduced states or free radicals are involved in the phytotoxic mode of action of DPEs. However, examination of the evidences in support of these possibilities reveals a number of difficulties. First, from a study of the redox properties of AFM, Orr *et al.* [26] concluded that mechanisms involving direct reduction and reoxidation of DPE molecule are probably not the basis for the action of these herbicides. On the other hand, Orr and Hogan [27] indicate that nitrofen can transfer electrons to an acceptor, either directly though an oxygen-independent mechanism or aerobically *via* superoxide radicals, and the formation of superoxide mediated by DPE radicals was further reported by Takahashi and Mason [28]. It nevertheless remains that, according to Gilham and Dodge [23], DPEs do not promote superoxide formation in illuminated chloroplasts, and the same conclusion has been reached concerning the pyridine LS 82-556 [17].

Moreover, formation of DPE radicals has been only observed on non-physiological conditions, *i.e.* under very strong illumination, and sometimes under UV light [24, 28, 29]. In consequence, these results give no clue as to whether DPE radicals really play some role *in vivo,* and their actual formation in treated plants is even questionable.

A third difficulty comes from the fact that the reduced or radical forms which could play a role in the mechanism of action of a DPE such as AFM involve the NO_2 substituent (Fig. 3). As pointed out by Orr *et al.* [26], replacing that substituent by a Cl atom does not change the mode of action of the molecule, and that obviously does not fit in with the above mechanism.

Nature of the photoreceptor

Let aside these problems concerning the redox and the radical theories, the nature of the photoreceptor must be considered. The pioneering studies of Matsunaka [2, 30], confirmed by Fadayomi and Warren [31], have shown that mutant plants devoid of chloroplastic pigments are tolerant to DPEs. Experiment with plants deprived of their pigments by pre-treatment with an inhibitor of carotenoid synthesis led to the same results [4, 32]. Moreover, Matsunaka [30] observed that mutants lacking both chlorophylls and carotenoids were tolerant to DPEs, while mutants lacking only chlorophylls were susceptible. From these observations, it was generally proposed that carotenes are the cellular photoreceptors responsible for the activation of DPEs [33].

Other experiments have brought additional pieces of information concerning that crucial point. It was found by Matringe *et al.* [4] that the pyridine LS 82-556, like DPEs, is inactive on plants devoid of pigments. Hence, depriving chloroplastic cells of their pigments makes these tolerant to DPE-type herbicides. That does not mean however, that chloroplastic pigments directly mediate the phytotoxic activity, because the absence of these pigments results in a number a secondary metabolic and structural changes, especially at the chloroplast level [34].

The possibility of a direct role of chlorophylls or carotenoids was further questioned by observations on cell cultures. It was found by Matringe and Scalla [10] that AFM and LS 82-556 are toxic to soybean cell cultures, and that the toxicity has the same characteristics as those established at the whole plant level, *i.e.* requirement for light and induction of lipid peroxidation resulting in cell lysis. Since these soybean cells are not chlorophyllous, any participation of photosynthesis is clearly excluded. On another hand, although devoid of chlorophylls, the cells contain some carotenoids. However, contrary to chlorophyllous tissues, they are killed by peroxidizing herbicides even if they have been deprived of their carotenes by treatment with an appropriate inhibitor [10]. Moreover, in these conditions, toxicity of peroxidizing herbicides can be induced by wavelengths in the blue region of the spectrum, *i.e.* in the zone where carotenoids absorb light. Similar observation have also been done by Gaba *et al.* [35], who observed that tomato cell cultures are killed by AF in the light even if they do not contain chlorophylls nor carotenoids. Finally, it has also been found that the phthalimide S-23142 is active on white seedlings of rice mutants or on cucumber seedlings with carotenoids depleted by fluridone [36].

Studies on the action spectrum of DPEs and similar herbicides

In spite of the wealth of informations concerning the possible formation of reduced or radical derivatives of DPEs and the importance of carotenoid pigments, it is difficult to draw firm conclusions about the mechanism of toxicity. It remains, however, that light is required by the phytotoxic process and that, at some point, a cellular photoreceptor is involved. In theory, it is possible to obtain indications on the nature of the photoreceptor by establishing a light action spectrum. That sort of experiment was done by Ensminger and Hess [37] on the alga *Chlamydonomas* treated by AFM, and led to the conclusion that chlorophylls and carotenoids were involved in herbicide toxicity. From a similar experiment on cucumber seedlings treated by AF-ethyl or the phthalimide S-23142, Sato *et al.* [38] could not draw clear conclusions, but suggested that multiple pigments, among which chlorophylls, were involved. These authors also put forward the hypothesis that peroxidizing herbicides cause formation of a photosensitizer by directly affecting molecules of chlorophylls or related pigments or by affecting their metabolic pathways.

These action spectra were established with chlorophyllous cells or tissues, so they could be obscured by interfering absorptions of various pigments not involved in the toxic process. For that reason, action spectroscopy was established with non-chlorophyllous tissues. Using that material, we found a maximum effect of AFM in the 350–450 nm region of the spectrum (Fig. 4). Wavelengths between 450 and 700 nm also induced toxicity but were less efficient [39, 40]. A very similar spectrum was found concerning the toxicity of LS 82-556 on etiolated cucumber hypocotyls [39]. Comparable conclusions were reached by Gaba *et al.* [35], who observed that 350 nm was the most effective wavelength inhibiting the growth of white

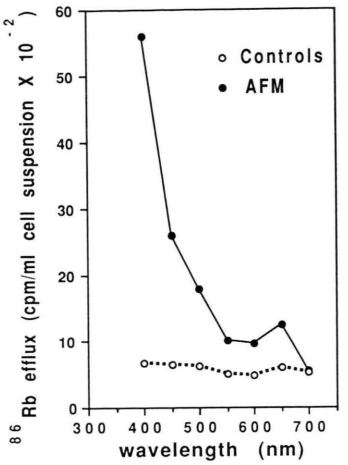

Fig. 4. Action spectrum of acifluorfen-methyl on non-chlorophyllous soybean cells. Redrawn from Matringe and Scalla [40].

tomato cells, and that red light was also effective, albeit to a lesser degree.

These action spectra obviously do not match with the absorption spectra of carotenoids, but rather suggest the participation of a tetrapyrrole pigment. At that point, it must be recalled that as shown by Rebeiz *et al.* [41], treatment of leaves by δ-aminolevulinic acid (ALA) plus an iron chelator causes an accumulation of tetrapyrrole intermediates of the chlorophyll biosynthetic pathway, that accumulation leading to a marked light sensitivity of the treated tissues.

Diphenyl ethers and related herbicides cause accumulation of protoporphyrin IX

The possibility of participation of a tetrapyrrole in the phytotoxicity of peroxidizing herbicides can be checked in several ways. First, it is possible to stop the biosynthesis of tetrapyrroles with an appropriate inhibitor. Indeed, Matringe and Scalla [39, 40, 43] have shown that 4,6-dioxoheptanoic acid, an inhibitor of ALA dehydratase [42] protects soybean cells and cucumber cotyledons against AFM [40, 43], and etiolated cucumber hypocotyls against LS 82-556 [39]. These results were confirmed by two other groups [9, 44, 45], who found that dioxoheptanoic acid and gabaculine, another tetrapyrrole synthesis inhibitor, protect plant tissues against various DPEs and oxadiazon.

A more direct demonstration for an interference with the metabolism of tetrapyrroles comes from the finding by Matringe and Scalla [39, 40, 43] that cells or tissues treated with AFM or LS 82-556 accumulate protoporphyrin IX (Proto IX), identified by its characteristic fluorescence and absorption spectra. Inducing Proto IX accumulation is a general property of DPEs and related herbicides, since it has been found by Lydon and Duke [44], Witkowski and Halling [45], and Sandmann and Böger [46], in cucumber, *Amaranthus retroflexus, Abutilon theophrasti* and the microalga *Bumilleriopsis filiformis,* treated with the following herbicides: AF (methyl), fluorodifen, oxyfluorfen, nitrofen, oxadiazon, LS 82-556 and chlorophthalim. That the accumulating chromophore is indeed Proto IX has been confirmed on several occasions [45–47]. At variance with these results, it has been reported by one group [48] that AFM induces, not only an accumulation of Proto IX, but also of protochlorophyllide.

Diphenyl ethers and related herbicides are inhibitors of protoporphyrinogen oxidase

Considering the biosynthesis pathway of chlorophylls and cytochromes (Fig. 5), accumulation of Proto IX can be explained by at least three possible mechanisms: stimulation of the biosynthesis of tetrapyrroles; inhibition of the insertion of metal atoms into Proto IX (*i.e.* inhibition of Fe and/or Mg-chelatases); or inhibition of protoporphyrinogen oxidase.

Taking into account the very fast accumulation of Proto IX, which can be detected after only 15 min of AF treatment, stimulation of tetrapyrrole synthesis is an attracting possibility, advocated by Kouji *et al.* [48]. In support of that mechanism, the authors present their above-mentioned evidence for accumulation of Proto IX *and* protochlorophyllide in AF-treated tissues. It must be emphasized, however, that only Proto IX has been found to accumulate upon herbicide treatment by Matringe et Scalla [40, 43], and that Witkowski and Halling [45] have ever found that AFM induces a loss of protochlorophyllide concomitant with the increase of Proto IX level.

The other possible sites of action of DPE-type herbicides, namely the chelatases and protoporphyrinogen oxidase, have been examined by Matringe *et al.* [49] and Witkowski and Halling [50].

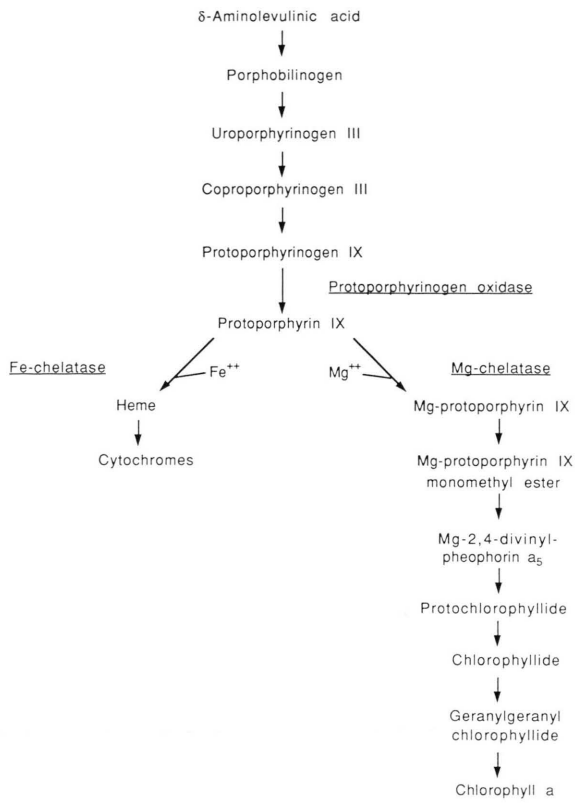

Fig. 5. Biosynthesis of chlorophylls and cytochromes.

The first experiments were based on the known ability of etioplasts to synthesize Mg-Proto IX from ALA or, more directly, from Proto IX. Results showed that AFM inhibits the formation of Mg-Proto from ALA, but much less from Proto IX, which means that it inhibits protoporphyrinogen oxidase, but has little activity on Mg-chelatase.

These conclusions have been confirmed by direct estimations of the effect of AFM on the relevant enzymatic activities [49]: AFM is a powerful inhibitor of protoporphyrinogen oxidase activity of corn etioplasts (I_{50} = 4 nM) and potato mitochondria (I_{50} = 0.43 nM). These figures are comparable to those of Witkowski and Halling [50], who found a I_{50} of 27 nM for the inhibition of the enzyme of cucumber chloroplasts. By contrast, AFM exerted a much weaker effect on the Fe-chelatase activity of potato mitochondria, with I_{50} above 100 μM [49].

Additional experiments have shown that AFM also strongly inhibits the protoporphyrinogen oxidase activity of yeast and mouse liver mitochondria, and that it is roughly 10,000 times less inhibitory to the corresponding Fe-chelatase activities [49]. And finally, inhibition of protoporphyrinogen oxidase appears to be a general property of various classes of light-requiring, DPE-type herbicides, since it has been demonstrated for oxadiazon, LS 82-556 and M & B 39 279 [51].

Generation of toxicity

Indeed, at first sight, the most obvious hypothesis accounting for Proto IX accumulation is that of an inhibition of chelatase, and especially of Mg-chelatases in chlorophyllous organs. As a matter of fact, that possibility has been initially put forward [9]. The enzymatic studies reported above have led to the surprising conclusion that Proto IX accumulates in consequence of protoporphyrinogen oxidase inhibition, or that, in other terms, inhibiting protoporphyrinogen oxidase actually *increases* the amount of the product of the reaction it normally catalyses. The same situation is found in the case of human diseases called *Porphyria variegata* [52], and of some yeast mutants [53]. In these cases, Proto IX accumulation results from a defect in protoporphyrinogen oxidase. It is generally assumed that unprocessed protoporphyrinogen molecules diffuse out of their site of synthesis, and then, owing to their known tendency to spontaneously oxidize, they react with molecular oxygen to give Proto IX. The same mechanism apparently takes place in plant cells treated with peroxidizing herbicides (Fig. 6).

Proto IX is a potent photosensitizer [54] which generates singlet oxygen in the light. Its uncontrolled accumulation thus accounts for the peroxidative degradation of cell lipids and the ensuing membrane lysis. It must be emphasized that the primary event in the action of DPE-type herbicides, *i.e.* the inhibition of protoporphyrinogen oxidase, occurs in the dark as well as in the light. The light-requiring step *i.e.* the generation of singlet oxygen by Proto IX, is only a secondary event resulting from the initial action of the herbicides.

Evidences in favor of the above mechanism

This mechanism is supported by a number of evidences, and in addition it throws a new light on

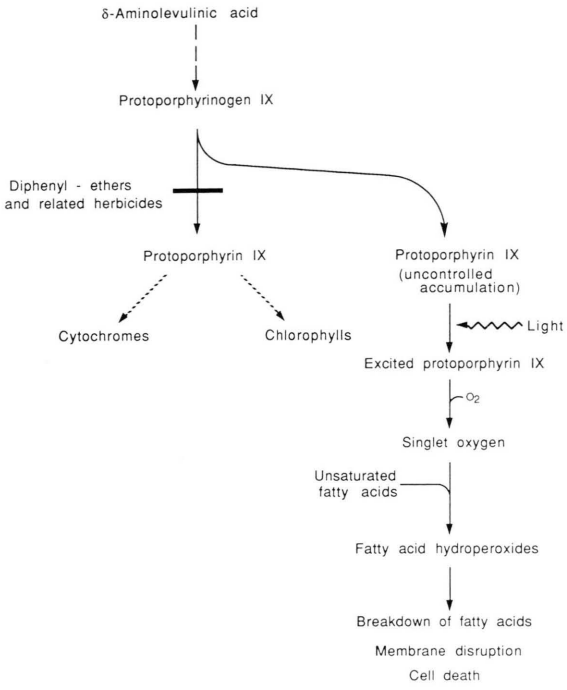

Fig. 6. Proposed scheme of the mechanism of action of diphenyl ethers and related herbicides.

some experimental facts the interpretation of which remained doubtful until recently.

First, as demonstrated by Lydon and Duke [44], Proto IX is indeed toxic to plant tissues: cotyledons incubated in darkness in a Proto IX solution exhibit electrolyte leakage upon exposure to light.

A second line of evidence is provided by the fact that all the treatments which alleviate the toxicity of peroxidizing herbicides also lower or suppress Proto IX accumulation, and *vice versa*. A first demonstration has been provided by the effect of inhibitors of tetrapyrrole biosynthesis, as explained earlier. Experiments with inhibitors of photosynthetic and respiratory electron flow have also led to the same conclusion. Matringe *et al.* [4] have shown that young cucumber plants are protected against AFM and LS 82-556 by pretreatment with diuron (or atrazine), and that there is no Proto IX accumulation in these conditions [43]. For an unknown reason, *detached* cotyledons are not protected by PS II inhibitors, and accordingly Proto IX accumulation, although lowered, is not suppressed in that case [43]. Non-chlorophyllous soybean cells can be protected against AFM and LS 82-556 with the respiration inhibitor antimycin A [10]. This protective effect is also correlated with a marked reduction in the Proto IX level [40].

The porphyrin theory also leads to a complete reappraisal of the lack of sensitivity of carotenoidless plants to DPEs. As a matter of fact, when carotenoid synthesis is inhibited by norflurazon, AFM or LS 82-556 induce no or very little Proto IX accumulation [43]. It is thus very likely that the tolerance of unpigmented plants is due, not merely to the absence of carotenes, but to a lack of porphyrin synthesis. It is known that carotenoid deficiency alters the structure and enzymatic content of chloroplasts [34], and we can assume that the porphyrin pathway is deficient in these abnormal organelles.

A last argument in support of the above presented mode of action comes from studies with AFM analogs (Fig. 7) [49]. As mentioned earlier, replacing the NO_2 group by a Cl atom to give LS 820340 lowers, but does not suppress, the light-dependent phytotoxic activity [26]. However, changing the position of the CF_3 substituent (RH 5348) almost abolishes the activity [18]. We have found [49] that the inhibitory powers of the three molecules on protoporphyrinogen oxidase of corn etioplasts and potato mitochondria parallel their phytotoxic abilities.

Conclusions

From the recent results we have exposed, it appears that the most satisfactorily explanation for the phytotoxicity of DPE-like herbicides is provided by their inhibiting protoporphyrinogen oxi-

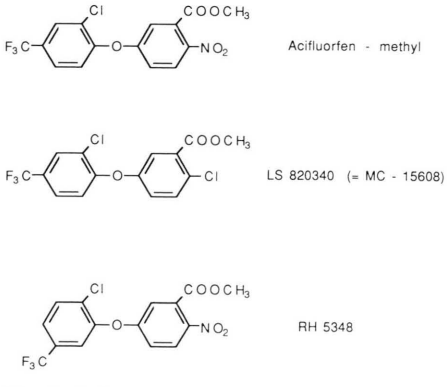

Fig. 7. Acifluorfen-methyl (AFM) and analogs.

dase. The efficiency of these molecules is due to several factors. First, they are potent inhibitors of the above enzyme. For example, I_{50} of AFM and oxadiazon are 4 nM and 11.5 nM respectively, for corn etioplast protoporphyrinogen oxidase, and 0.43 nM and 9 nM respectively, for the enzyme of potato mitochondria [49, 51]. Moreover, it is conceivable that only a partial inhibition of protoporphyrinogen oxidase activity is sufficient to kill a plant cell, since it probably results in a sizeable accumulation of Proto IX.

With regard to future studies, some points remain to be more precisely elucidated. First, it has been shown in some cases that the extent of cellular damages correlates positively with the amount of Proto IX induced to accumulate. That relation has been demonstrated for cucumber cotyledons treated with AF or oxadiazon [55], and for soybean cell culture treated with oxyfluorfen [56]. The relation is much less clear, however, for some microalgae [46]. Also in the case of soybean *in vitro* cultures treated with nitrofen we have found that cell lysis is only accompanied by a barely detectable Proto IX accumulation [56]. Several factors could go to explain these puzzling results: first, some microorganisms apparently excrete Proto IX under an inactive (agregated) form in the external medium, in which it is no longer harmful [46, 53, 57]. Second, Proto IX is prone to undergo photochemical autooxidation, and thus self-destroys in the light [55]. It follows that accumulation of Proto IX can be more conveniently followed in the dark, whereas toxicity has to be estimated upon exposure to the light, which makes experimental results sometimes difficult to be interpreted.

A final remark concerns the surprising speed at which Mg-Proto accumulates upon herbicide treatment. That might suggest that some regulatory mechanism no longer operates. It is known, for example, that formation of ALA from glutamate is inhibited *in vitro* by heme and Mg-Proto [61]. Thus an arrest of the synthesis, or an abnormal subcellular localization of these compounds may result in an uncontrolled synthesis of tetrapyrroles. In order to reach a definitive answer on this point, it will be necessary to understand the respective roles of chloroplasts and mitochondria in the formation of Proto IX. Isolated greening plastids are capable of forming both heme and chlorophyll from glutamate [59], but the synthetic capacities of plant mitochondria are much less clear. In maize, even the mitochondrial heme *a* (which is the prosthetic group of Cyt oxidase) is made from ALA that is synthesized from glutamate, and not by condensation of succinyl-Co A and glycine catalyzed by ALA synthase as in animal mitochondria [60]. One possibility is that the first steps of tetrapyrrole synthesis, up to protoporphyrinogen, take place in the chloroplast. The transfer of protoporphyrinogen to mitochondria could thus be a critical point, prone to lead to Proto IX accumulation upon inhibition of protoprophyrinogen oxidase. That possibility however, is presently purely speculative, and further research is clearly needed in this area.

Acknowledgements

We thank Jean-Claude Gaudry for his skillful preparation of the figures.

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