Binding and Peroxidative Action of Oxyfluorfen in Sensitive and Tolerant Algal Species

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Peroxidative activity of oxyfluorfen and binding of this nitrodiphenyl ether to cell fractions was investigated with the susceptible alga *Scenedesmus acutus* and the resistant alga *Bumilleriopsis filiformis*. Although a 10-fold higher concentration of oxyfluorfen was applied to *Bumilleriopsis*, the lag phase for initiation of peroxidative evolution of short-chain hydrocarbons from fatty acids was much longer than found with *Scenedesmus*. Oxyfluorfen was predominantly recovered after homogenization from the pellet which was separated into a lipid and a chloroform/methanol insoluble fraction. Parts of the oxyfluorfen which is present in the insoluble pellet fraction during the lag phase before the onset of peroxidation can be found in the lipid fraction when measurable peroxidative activities have started. This was observed with *Scenedesmus* as well as with *Bumilleriopsis*. During peroxidation initiated by oxyfluorfen acyl lipids are degradated as monitored by the disappearance of the plastidic sulfolipid. Analysis of bound fatty acids showed that they are targets for peroxidative reactions in acyl lipids. Destruction of polyunsaturated fatty acids was higher than for saturated ones.

Introduction

p-Nitrodiphenyl ethers like oxyfluorfen act predominantly as peroxidative herbicides in the light [1]. Either chlorophylls in connection with photosynthetic electron transport or carotenoids were suggested as light receptors [2, 3]. There is evidence by EPR measurement [4] and studies with radical quenchers such as vitamin E [5, 6] or ethoxyquin [7] that nitrodiphenyl ethers initiate a radical chain reaction in the light which leads to the destruction of cell components [8]. Prominent targets are the polyunsaturated fatty acids of membrane lipids. Detectable reaction products of fatty acid peroxidation are volatile hydrocarbons of variable chain length which is determined by the position of the double bonds in the fatty acid molecule [9].

In higher plants tolerance against nitrodiphenyl ethers has been reported for several species according to their endogenous levels of the antioxidants vitamin C and E [10]. A similar variation in sensitivity against oxyfluorfen could also be observed for different microalgae [2, 9].

This study uses two algal species, *Scenedesmus* which shows high sensitivity towards oxyfluorfen [2] and *Bumilleriopsis* which is tolerant to a certain extent [11]. In both algae binding of [^{14}C]oxyfluorfen to cell fractions and peroxidative hydrocarbon evolution was compared. Furthermore, determination of a specific plastidic sulfolipid, sulfoquinovosyl diglyceride, and analysis of fatty acids were performed with *Bumilleriopsis* under peroxidizing conditions.

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Materials and Methods

Scenedesmus acutus [2] and *Bumilleriopsis filiformis* [11] were cultivated at a light intensity of 30 W/m^2 as previously described. Packed cell volume was determined in graduated microcentrifuge tubes of 80 µl capacity. Measurement of saturated short chain hydrocarbons (ethane, propane, and pentane) was performed with 2 ml samples (cell density of 2 µl/ml pcv) placed into 6 ml head space vessels. Gas samples were automatically withdrawn and injected into the gas chromatograph. Details of this procedure and determination of short chain hydrocarbons are given in ref. [9].

Scenedesmus and *Bumilleriopsis* cells were treated with 0.4 µCi oxyfluorfen (final concentration 1 and 10 µM, respectively). Aliquots were withdrawn and analyzed for oxyfluorfen incorporation. Cells were harvested by centrifugation, washed with 0.2 M Tris-HCl buffer, pH 7.5, resuspended in the same buffer, and broken with glass beads (0.55 mm diameter) in a Bühler Vibrogen cell mill. After removal of the glass beads, the homogenate was centrifuged at 43,000 × *g*

for 30 min. The "soluble protein fraction" was obtained by adding equal amounts of 20% TCA in ethanol (w/v) to the supernatant. The resulting precipitate was dissolved in Soluene (purchased from Packard) and subjected to liquid-scintillation measurement. The membrane pellet was extracted with hot methanol (15 min, 65 °C) and subsequently with chloroform/methanol for 15 min at 55 °C. The colored extracts were pooled and the radioactivity of this "lipid fraction of the pellet" determined after photobleaching of the chlorophylls. The membrane residues, the "insoluble pellet fraction", was dissolved in Soluene and counted for radioactivity.

Oxyfluorfen-dependent lipid degradation in *Bumilleriopsis* was followed by tracing the ^{35}S-prelabeled plastidic sulfolipid (sulfoquinovosyl diglyceride). After 48 h of oxyfluorfen treatment, cells were harvested and the sulfolipid was extracted and separated by TLC. The disappearance of the sulfolipid was determined by ^{35}S-radioactivity. The details of this procedure are given in ref. [12]. Chlorophyll was quantitated after hot methanol extraction (15 min, 65 °C) according to MacKinney [13].

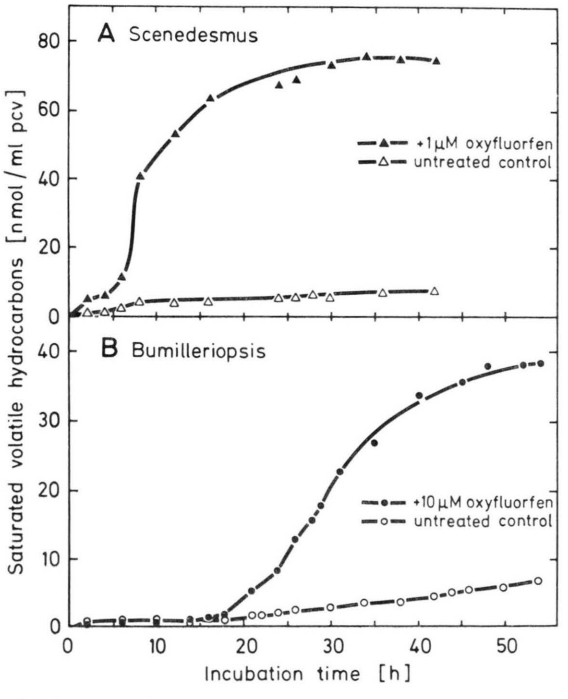

Fig. 1. Peroxidative formation of short chain saturated hydrocarbons in the presence of oxyfluorfen in cultures of *Scenedesmus* (A) and *Bumilleriopsis* (B).

Analysis of membrane bound fatty acids from *Bumilleriopsis* cultures (48 h growth) was performed after methanol/chloroform extraction and transesterification with BF_3/methanol. The fatty acid methylesters were separated, identified and quantitated by GC on a 15% DEGS column at 180 °C (see ref. [11] for details).

Results

The algae *Scenedesmus* and *Bumilleriopsis* exhibit different kinetics in peroxidative formation of hydrocarbon gases initiated by oxyfluorfen (Fig. 1). Both curves show a lag phase after oxyfluorfen application which lasts either about 5 h for *Scenedesmus* or about 20 h for *Bumilleriopsis*, although the oxyfluorfen concentration was 10-fold higher in the latter culture. Saturation of short-chain hydrocarbon formation was reached about 30 h after the onset of gas production in both cases.

The two algae were assayed for distribution of [^{14}C]oxyfluorfen in different biochemical fractions, in a TCA supernatant, in soluble proteins and in a lipid fraction as well as an insoluble pellet (Fig. 2). In case of *Scenedesmus* analyses were made after a 2 h oxyfluorfen treatment – when a peroxidative effect was not yet evident – and after a 5 h treatment, when peroxidation had already started. *Bumilleriopsis* cells were assayed after treatment for 3 and 24 h with oxyfluorfen to account for similar non-peroxidative and peroxidative conditions. In addition, the oxyfluorfen concentrations differed as was the case in Fig. 1, namely 1 µM in the *Scenedesmus* and 10 µM in the *Bumilleriopsis* culture. In all samples the majority (about 80%) of the radioactivity from oxyfluorfen was recovered in the lipids and the insoluble fraction of the pellet. Parallel experiments with heterotrophically (dark)-grown *Scenedesmus* gave the same distribution of oxyfluorfen in the various fractions.

Prior to the onset of peroxidative hydrogen gas production, *i.e.* in cell material analyzed after 2 and 3 h, respectively, the oxyfluorfen distribution in the insoluble pellet fraction compared to the lipids was always found higher than under subsequent peroxidative conditions. After the onset of peroxidative hydrocarbon gas production, oxyfluorfen-binding to the lipophilic pellet fraction increased whereas the radioactivity in the insoluble pellet fraction simultaneously decreased. This shift of oxyfluorfen recovery

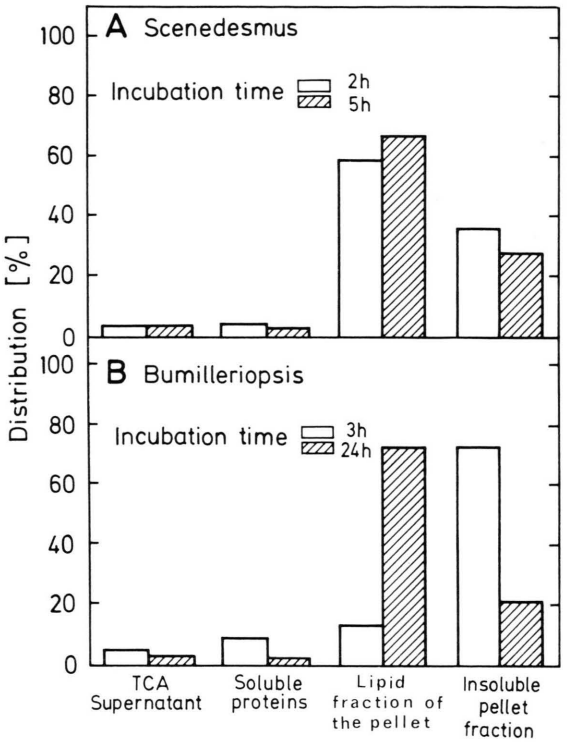

Fig. 2. Incorporation of [^{14}C]oxyfluorfen and distribution in different cell fractions during peroxidative lag phase or under peroxidative conditions. (A) *Scenedesmus* cultures and (B) *Bumilleriopsis* cultures treated with 1 μM or 10 μM oxyfluorfen, respectively. Total incorporation of [^{14}C]oxyfluorfen per 600 ml algal suspension was 850,000 dpm in (A) and 780,000 dpm in (B).

from the insoluble pellet fraction to the lipid fraction is very strong in *Bumilleriopsis* but not as extremely pronounced in *Scenedesmus*.

As oxyfluorfen is predominantly associated with the lipids of the pellet when peroxidation is in progress, concurrent peroxidative destruction of a selected membrane lipid, the plastidic sulfolipid, was determined in *Bumilleriopsis* (Table I). After washing the cells and transfer into media with unlabeled sulfate, radioactivity in the ^{35}S-prelabeled sulfolipid was retained in the control culture for 48 h. The presence of 10 μM of oxyfluorfen resulted in a 56% decrease of this lipid. The chlorophyll content of the control culture increased about 3-fold over 48 h whereas in the oxyfluorfen-treated culture only a 1.2-fold increase of total chlorophyll per culture could be observed.

The lipids of *Bumilleriopsis* contain a range of even-numbered fatty acids with 14 to 20 carbon atoms with up to 5 double bonds (Fig. 3). In addition to the common ω-3 and ω-6 fatty acids, a unique 16:3 ω-4 fatty acid could be demonstrated in *Bumilleriopsis*. All the fatty acids found were affected by oxyfluorfen. Destruction of the various fatty acids was between 20 and 55%. The decrease was lowest for the saturated fatty acids and comparably high for the fatty acids with 4 and 5 double bonds.

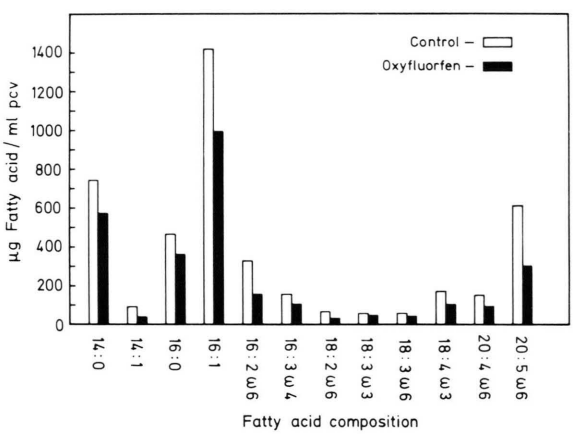

Fig. 3. Pattern of bound fatty acids in cultures of *Bumilleriopsis* after a 48 h treatment with 10 μM oxyfluorfen.

Table I. Degradation of sulfolipid and chlorophyll of *Bumilleriopsis* in the presence of 10 μM oxyfluorfen.

	Culture directly after inoculation	Culture after 48 h	
		Control	+ Oxyfluorfen
Radioactivity in sulfolipid per culture (10^3 dpm/l suspension)	256	230	128
Chlorophyll per culture [mg/ml]	39	101	46

Discussion

Oxyfluorfen initiates peroxidation in *Scenedesmus* and *Bumilleriopsis*, but sensitivity of both species is quite different (Fig. 1). One indication of a weaker peroxidative response by *Bumilleriopsis* is a much longer lag phase than in *Scenedesmus*, even at a 10-fold higher oxyfluorfen concentration. Uptake and incorporation of oxyfluorfen into the algal cells was the same after 2 and 5 h or 3 and 24 h, respectively (Fig. 2 A and B). Therefore, it can be concluded that the lag phase is not caused by slow oxyfluorfen uptake but rather accounts for the period that the antioxidative system can cope with peroxidation before it is overtaxed. It has been demonstrated with mustard seedlings that degradation of antioxidant vitamin C preceeds peroxidative ethane evolution in the presence of paraquat [14]. Determination of endogenous vitamin C has shown that the tolerant *Bumilleriopsis* cells contain a 10-fold higher level than *Scenedesmus* (Dr. K.-J. Kunert, personal communication). Therefore, the early oxyfluorfen tolerance of *Bumilleriopsis* can be explained by its more efficient protection system against peroxidative damage.

About 80% of the oxyfluorfen incorporated into *Scenedesmus* and *Bumilleriopsis* is found in the insoluble fraction of the pellet which contains membranes and cell walls (Fig. 2). When antioxidants no longer prevent peroxidative hydrocarbon formation, after 3 h in *Scenedesmus* and after 18 h in *Bumilleriopsis*, the oxyfluorfen accumulation shifts from the insoluble pellet fraction into the lipids. This shift is especially pronounced in the tolerant *Bumilleriopsis*.

It was previously proposed that diphenyl ether herbicides become free radicals during light activation [5, 8] and recent EPR measurements support this view [15]. This possibility could explain the preferential binding of oxyfluorfen to the lipid fraction during progressing peroxidation, especially in *Bumilleriopsis*. It is well known that potential targets of radicals are the double bonds of fatty acid moieties of acyl lipids [8, 16]. Acyl lipids and prenyl lipids are preferentially destroyed in peroxidative reactions [17]. As was shown previously [18] with isolated thylakoids from spinach, a 15 min light incubation with oxyfluorfen led to tight binding since only in the dark experiment [^{14}C]nitrofen could be replaced by the unlabeled herbicide.

Degradation of acyl and prenyl lipids could be directly demonstrated for *Bumilleriopsis* treated with oxyfluorfen for 48 h (Table I). The destruction of sulfoquinovosyl diglyceride can be taken as an indication that other acyl lipids (*e.g.* monogalactosyl and digalactosyl diglyceride) are also affected. Analysis of the fatty acid components of these membrane lipids demonstrates that oxyfluorfen-dependent peroxidation destroys these fatty acids, especially the polyunsaturated ones (Fig. 3).

Our studies with oxyfluorfen-susceptible and -tolerant algal species provide another line of evidence for the following proposed mechanism concerning the peroxidative mode of action of diphenyl ethers: Oxyfluorfen is converted to a radical by light activation. During a lag phase, these radicals are quenched by the antioxidative systems. Consequently, tolerance against peroxidative diphenyl ethers depends on the amounts of antioxidants available. In the subsequent phase of peroxidation, the diphenyl ether radical attacks and binds to lipids which results in peroxidative destruction of fatty acids bound to acyl lipids.

Acknowledgements

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