

The Diphenyl-Ether Herbicide Oxyfluorfen: A Potent Inducer of Lipid Peroxidation in Higher Plants

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Measurements of ethane and ethylene as indices of *in vivo* lipid peroxidation and of chemically-induced stress, respectively, were done on mustard seedlings (*Sinapis alba*), treated with oxyfluorfen[2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene]. When seedlings were exposed to low light intensity of 14 W/m², only stress-ethylene production but not peroxidative ethane evolution was significantly higher over 10 days in herbicide-treated seedlings than in the untreated control. Exposure to high light intensity of 390 W/m² did not increase stress-ethylene production in oxyfluorfen-treated plants after 24 h. Lipid peroxidation, however, measured as ethane evolution, was substantially higher after 24 h in herbicide-treated plants, exposed to high light intensity, than in the control, and peroxidation was directly related to strong oxidation of antioxidants and to severe membrane damage.

Introduction

Diphenyl ether herbicides exhibit a broad spectrum of weed control in several crops [1]. Since 1974, the *p*-nitrodiphenyl ether oxyfluorfen [2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene] has been experimentally used in soybeans for the control of grasses and troublesome annual broadleaf weeds, such as black nightshade (*Solanum nigrum*) and morning glory (*Ipomoea* spp.). Both light and oxygen play important roles in the activity of oxyfluorfen and several other *p*-nitrodiphenyl ethers [2–5]. It has been reported that oxyfluorfen becomes more active as light intensity increases and a hyperbolic relationship between both has been found [6].

In vivo lipid peroxidation is one of the most deteriorative reactions basic to the mechanism of membran and cellular damage [7]. Membranes, especially those of subcellular organelles, are the major sites of damage caused by lipid peroxidation within the cell. These membranes contain relatively large amounts of polyunsaturated fatty acids that can be peroxidized [8]. Experimental work has provided evidence for the occurrence of destructive peroxidation reactions as a phytotoxic consequence of herbicidal action. One line of evidence has been the measurement of oxyfluorfen-induced membrane degradation [5, 6, 9–11]. A second line has been the identification of the hydrocarbon gas ethane [12, 13],

and a third line of evidence has been the protection of herbicide-treated cells by antioxidants *in vivo* [5, 14].

Research to date has shown that vitamins E and C protect against peroxidation reactions *in vivo*. Vitamin C has been proposed as an antioxidant-synergist with vitamin E [15, 17]. Vitamin E acts as the primary antioxidant, while vitamin C reductively regenerates oxidized vitamin E. The interaction of both vitamins has been observed by pulse radiolysis techniques [18].

Little is known, however, about the antioxidative role of the vitamins in higher plants. Recently, we reported that herbicide-induced ethane evolution, as an index of *in vivo* lipid peroxidation, seemed to be a function of the vitamin-C concentration in plants [19].

In this study, we have demonstrated some additional aspects of oxyfluorfen-induced lipid peroxidation in mustard seedlings (*Sinapis alba*). The influence of light intensity on the magnitude of the peroxidation process and the extent of both antioxidant and membrane damage was determined. The sensitive measurement of evolved ethane, a decomposition product of ω 3-unsaturated fatty-acid hydroperoxides, was used to show *in vivo* lipid peroxidation after herbicide treatment.

Materials and Methods

Mustard seeds were planted in plastic pots containing vermiculite. Seedlings were grown for

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10 days in a growth chamber regulated to a 12 h photoperiod (14 W/m^2 additional light) with day and night temperatures of 22°C and 18°C , respectively. Seedlings that had developed the cotyledons after 10 days were sprayed with an emulsifiable concentrate formulation of oxyfluorfen mixed with water. After herbicide treatment, seedlings were either continuously illuminated for 24 h with light of 390 W/m^2 (high light intensity) at 22°C , or illuminated for 10 days with light of 14 W/m^2 (low light intensity) in a growth chamber regulated to the day and night conditions described above.

Evolution of hydrocarbon gases was measured by gas chromatography by a modified procedure of Dillard *et al.* [20]. Volatile hydrocarbons were analyzed on a Perkin-Elmer F22 gas chromatography equipped with a flame ionization detector and fitted with a Varian six-way gas sample valve. A stainless steel column ($0.32 \text{ cm} \times 152 \text{ cm}$), filled with activated alumina (80 to 100 mesh), was used with a nitrogen carrier-gas flow rate of 30 ml/min . The column temperature was 60°C and the temperature of both injector and detector was 200°C .

For sample collection, hydrocarbon-free air (methane $\leq 0.1 \text{ ppm}$; Messer Griesheim GmbH, Düsseldorf, Germany) was passed through a dust filter. The air then passed at a flow rate of 10 l/h through a flowmeter and into the plant chamber. The chamber was constructed of two identical polyacrylamide cylinders that could be connected by wing nuts, and could be disconnected from the sample collecting system in order to accumulate volatile hydrocarbons evolved by the plant seedlings for 30 min. The air stream passed on the effluent side of the chamber through a plastic column ($2 \text{ cm} \times 10 \text{ cm}$) that contained equal parts of Ascarite and Sikkon-blue (both chemicals from Fluka AG, Buchs SG, Switzerland) to remove carbon dioxide and water vapour, respectively. The air stream was then split to obtain a flow rate of 5 l/h through each of two flowmeters. The effluent side of one flowmeter was connected to the six-way gas sample valve whereas the other flowmeter was always open to prevent a pressure increase in the plant chamber. Attached to the gas sample valve was a stainless steel loop (20 cm) that contained activated alumina (80–100 mesh) in the lower 7 cm of the loop. During collection the trap was cooled down in an ethanol liquid-nitrogen bath. The gas (500 ml) which had been accumulated in the chamber was

collected in a vacuum flask which attached to the gas sample valve and a calibrated mercury manometer. After collection of the sample, the trap was heated to 70°C in a water bath and the sample injected from the trap onto the gas chromatographic column *via* copper tubing.

The relative peak areas of hydrocarbons measured from samples were calculated by comparison with an 1 ppm ethane standard (Messer Griesheim GmbH, Düsseldorf, Germany), injected directly *via* a 1 ml gas-tight syringe into the gas chromatograph.

Total carotenoids were determined by the method of Goodwin [21]. Cotyledons (2 g) were homogenized in 80% acetone containing 1% NaOH. After extraction with petroleum ether (b.p. $40\text{--}60^\circ\text{C}$), the absorbance of the yellow extract was measured at 440 nm in a spectrophotometer, and the amount of total carotenoids calculated using an extinction coefficient of 2500 for a 1% solution of carotenoids.

The vitamin-C content of cotyledons was determined by HPLC using paired ion reversed phase chromatography according to the method of Finley and Duang [22]. Cotyledons (2 g) were homogenized in a 2% solution of metaphosphoric acid containing 2 mM EDTA. For vitamin-C determinations, clear supernatants ($20 \mu\text{l}$) were introduced after centrifugation onto a LiChrosorb RP 18 HPLC-column and analyzed.

All estimates of sample variability are given in terms of the standard error (SE) of the mean. Comparisons of two means were calculated using Student's two-tailed *t*-test. *p*-values ≤ 0.05 were considered significant.

Results

The dry weight calculated as percentage of the fresh weight of cotyledons was significantly higher ($p < 0.01$) for mustard seedlings treated for 10 days with oxyfluorfen under low light intensity than in the control. Within 10 days, seedlings developed a chlorotic appearance of the leaf tissue. Exposure to light of high intensity almost totally damaged the oxyfluorfen-treated plants and the percentage of dry weight increased to 43%. This value was significantly higher ($p < 0.01$) than either the 12% and 17% for the control and for herbicide-treated plants grown under low light intensity, respectively. When seedlings were exposed to the dark, no toxic in-

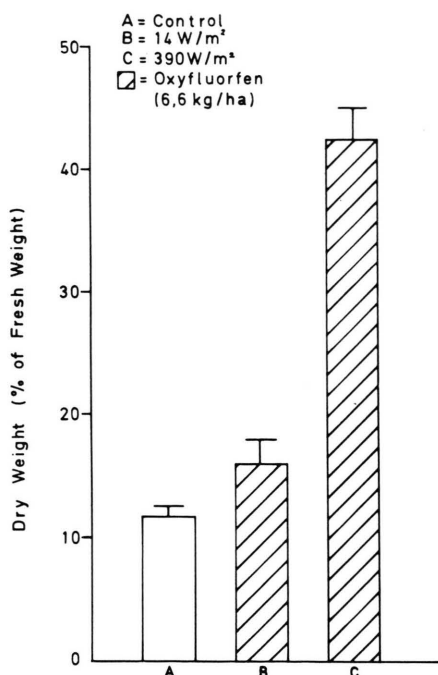


Fig. 1. Dry weight calculated as percentage of fresh weight of untreated mustard seedlings (A) and seedlings treated with 6.6 kg/ha of oxyfluorfen under low light intensity of 14 W/m² (B) for 10 days or for 24 h under high light intensity of 390 W/m² (C). For both light conditions an identical percentage value was found for control plants. Data shown represent the mean \pm SE of 15 plants in both the control group and the group grown under high light intensity, and 10 plants in the group grown under low light intensity.

fluence of the herbicide was observed (data not shown).

Compared to the untreated control, application of oxyfluorfen significantly increased ($p < 0.05$) ethylene production within 10 days in seedlings cultivated under low light intensity (Fig. 2). However, when herbicide-treated plants were exposed for 24 h to high light intensity, the mean value for the amount of the hydrocarbon gas evolved was lower than in the control. After 10 days, ethane production of both oxyfluorfen-treated seedlings grown under low light intensity and untreated plants was not significantly ($p > 0.05$) different, although the mean of ethane evolution was 1.5-fold higher in herbicide-treated plants than in the control (Fig. 2). Ethane evolution dramatically increased, however, when seedlings were illuminated after oxyfluorfen application with light of high intensity. After 24 h, herbicide-treated plants had a 19-fold higher ethane production ($p < 0.01$) than the control.

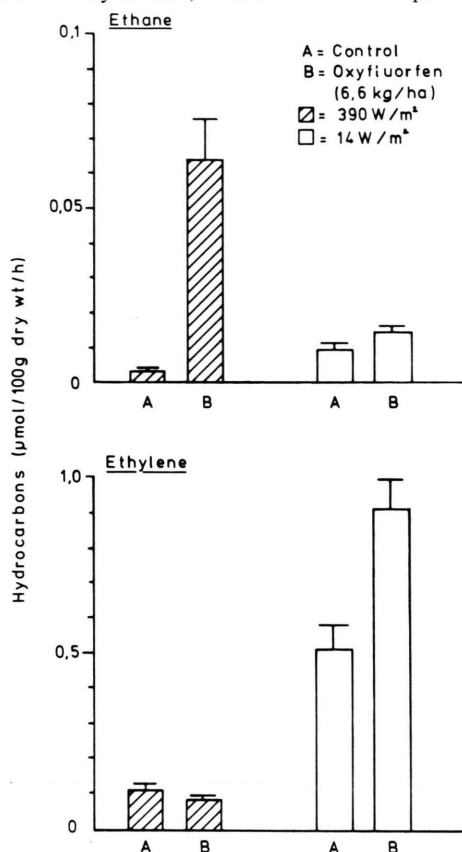


Fig. 2. The amount of ethane and ethylene produced by untreated mustard seedlings (A) and seedlings treated with 6.6 kg/ha of oxyfluorfen (B) cultivated either under low light intensity of 14 W/m² for 10 days or for 24 h under high light intensity of 390 W/m². Data shown represent the mean \pm SE of 10 plants in both the control group and the group grown under high light intensity, and 6 plants in the group under low light intensity.

The amounts of both vitamin C and carotenoids were significantly lower ($p < 0.01$) in oxyfluorfen-treated plants either cultivated under low light intensity or high light intensity than in the untreated control (Fig. 3). Application of the herbicide decreased the content of carotenoids approximately 53% under both light conditions. However, vitamin-C decrease was 2.5-fold greater ($p < 0.05$) in seedlings grown under high light intensity than in seedlings grown under low light intensity.

Discussion

Among the toxic compounds that can initiate *in vivo* lipid peroxidation, certain bleaching herbicides, such as the diphenyl ether oxyfluorfen, have been

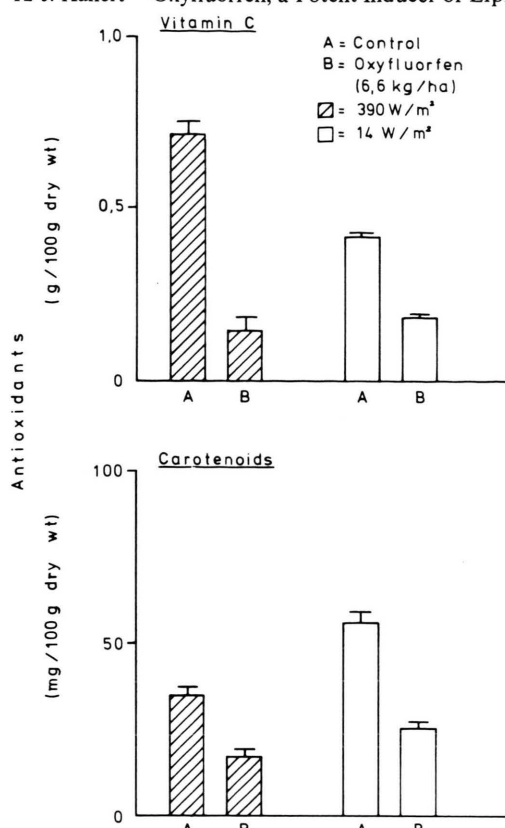


Fig. 3. The amount of vitamin C and carotenoids in untreated mustard seedlings (A) and seedlings treated with 6.6 kg/ha of oxyfluorfen (B) cultivated either under low light intensity of 14 W/m² for 10 days or for 24 h under high light intensity of 390 W/m². Data shown represent the mean \pm SE of 6 plants in each group.

identified [12, 13]. In general, peroxidation is initiated by an prooxidant, and semi-stable hydroperoxides, from free radical intermediates, are formed by the reaction of molecular oxygen with polyunsaturated fatty acids [7]. A proposed pathway for part of the reaction under study here is shown in Figure 4. Neither the activation of the herbicide nor the mechanism by which oxyfluorfen mediates lipid peroxidation is yet known with certainty. In this issue, there is a report by Böger discussing the activation and the prooxidative nature of *p*-nitrodiphenyl ethers in more detail.

Formation of volatile hydrocarbons from the decomposition of lipid hydroperoxides is well documented [23]. Many studies have shown ethane, a decomposition product of ω 3-unsaturated lipid hydroperoxids, to be a useful index of *in vivo* lipid

peroxidation [7, 16, 24]. In our studies, we have successfully used this noninvasive methodology of ethane measurement to show oxyfluorfen-induced lipid peroxidation in the intact plant. Previously, it had been reported that the herbicide becomes more active as light intensity increases [6]. We have been able to confirm this result. In our experiments, the peroxidation process was totally light dependent, and the amount of ethane produced by herbicide-treated seedlings was controlled by the light intensity used. Furthermore, the increase of hydrocarbon-gas evolution was directly related to a dramatic increase of the dry weight, calculated as percentage of fresh weight. These data strongly support the hypothesis that peroxidative membrane damage is a severe phytotoxic consequence of herbicidal action under high light intensity.

Under low light intensity lipid peroxidation was no longer the dominant reaction in our system. Under these conditions, the production of ethylene was significantly stimulated after the application of oxyfluorfen. There is a substantial amount of evidence that the two volatile hydrocarbons, ethylene and ethane, are produced by different pathways. Methionine is the biological precursor of ethylene whether it is produced under normal or stress conditions [25], while ethane is only derived from the peroxidation of ω 3-polyunsaturated fatty acids [7]. It is well documented that toxic compounds, such as herbicides, affect chemical-induced stress ethylene production by plant tissues [10, 26] and herbicidal ethylene evolution is strongly reduced by aminoethoxyvinylglycine (AVG), a specific inhibitor of ethylene formation from methionine [26, 27]. The magnitude of gas evolution is determined by the extent of stress or damage undergone by plant tissues. Accelerated senescence is apparent as one physiological consequence of increased ethylene production [28].

In contrast to low-intensity light, light of high intensity was found to strongly prevent ethylene production in oxyfluorfen-treated seedlings. Our result is consistent with that of Elstner and Konze [29]. Working with point frozen leaf discs, they observed that ethylene evolution declined when stress resulted in severe cell damage and peroxidation of lipids became significantly evident. It is known that toxic products are formed during lipid peroxidation and decomposition of these products can cause protein damage by cross-linking reactions

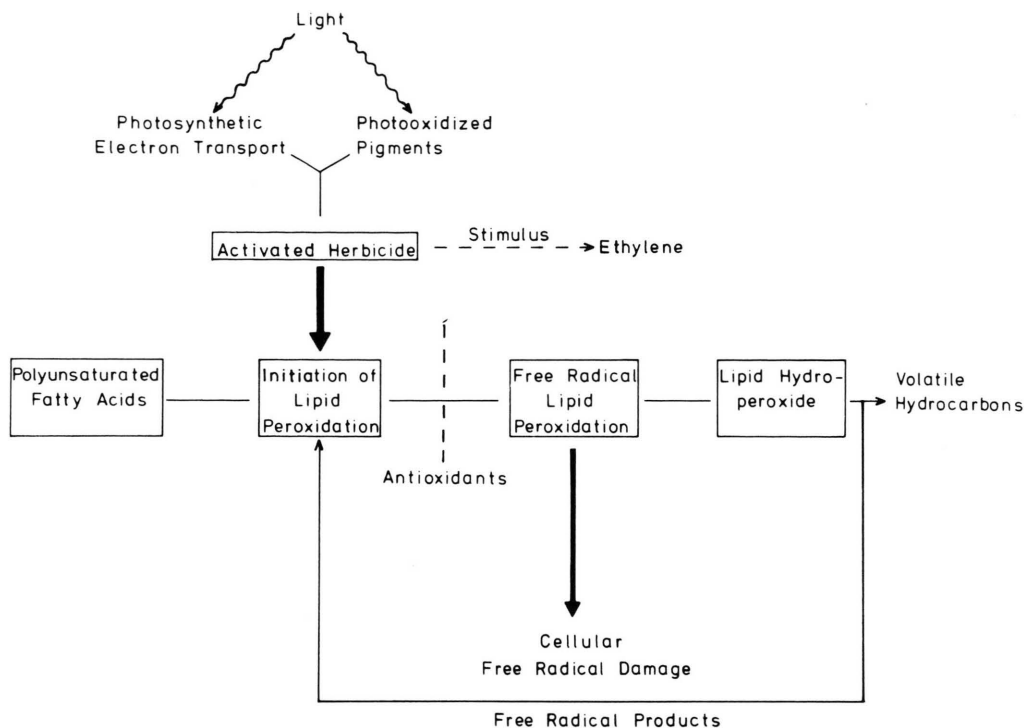


Fig. 4. Proposed pathways for reactions involving activation of the diphenyl ether oxyfluorfen [5, 12], lipid peroxidation, and the action of antioxidants.

[30] or damage of the prosthetic group of photosynthetic active proteins [31]. In our experiments, a similar process also seems to be responsible for the decline of ethylene production because research has shown that ethylene biosynthesis is regulated by an enzyme system that converts methionine to ethylene [25].

When lipid peroxidation was the dominant reaction in our system, natural antioxidants, such as vitamin C and carotenoids, were oxidized. However, the oxidation of vitamin C was greater than that of carotenoids. Co-oxidation of antioxidants is known to be part of the physiological defense system against the deteriorative peroxidation process. A sufficient amount of reduced antioxidants has to be present in the cell to terminate free radical reactions by hydrogen abstraction [7, 32]. A low antioxidant concentration can strongly potentiate the effect of an initiator on lipid peroxidation [16]. Working with peroxidative herbicides, we have recently reported a highly significant correlation in plants between both the decrease of vitamin C and the evolution of

ethane, and we have strong evidence that herbicide-mediated peroxidation seems to be a function of the antioxidant concentration in plants [19, 33]. More research is required, however, to support our hypothesis that herbicide toxicity is highly controlled by natural protectors of *in vivo* lipid peroxidation.

Overall, lipid peroxidation is the dominant reaction in oxyfluorfen-treated mustard seedlings only under conditions of high light intensity. Under low light intensity, however, herbicide treatment results in stress ethylene production. The light-induced peroxidation process is directly related to a strong oxidation of antioxidants and to severe membrane damage that results in cell death.

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