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Effects of Acifluorfen on Endogenous Antioxidants and Protective Enzymes in Cucumber (*Cucumis sativus* L.) Cotyledons

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ABSTRACT

The herbicide acifluorfen (2-chloro-4-(trifluoromethyl)phenoxy-2-nitrobenzoate) causes strong photooxidative destruction of pigments and lipids in sensitive plant species. Antioxidants and oxygen radical scavengers slow the bleaching action of the herbicide. The effect of acifluorfen on glutathione and ascorbate levels in cucumber (*Cucumis sativus* L.) cotyledon discs was investigated to assess the relationship between herbicide activity and endogenous antioxidants. Acifluorfen decreased the levels of glutathione and ascorbate over 50% in discs exposed to less than 1.5 hours of white light (450 microeinsteins per square meter per second). Coincident increases in dehydroascorbate and glutathione disulfide were not observed. Acifluorfen also caused the rapid depletion of ascorbate in far-red light grown plants which were photosynthetically incompetent.

Glutathione reductase, dehydroascorbate reductase, superoxide dismutase, ascorbate oxidase, ascorbate free radical reductase, peroxidase, and catalase activities rapidly decreased in acifluorfen-treated tissue exposed to white light. None of the enzymes were inhibited *in vitro* by the herbicide. Acifluorfen causes irreversible photooxidative destruction of plant tissue, in part, by depleting endogenous antioxidants and inhibiting the activities of protective enzymes.

The herbicide acifluorfen is highly effective in the selective control of a wide spectrum of broadleaf weeds in a number of major crops, *e.g.* soybeans. Although increasing emphasis has been placed on discerning the mechanism of action of this herbicide recently, a precise understanding of how acifluorfen causes phytotoxicity has remained elusive. Light is an absolute requirement for herbicidal activity of acifluorfen and other closely related *p*-nitro DPE[2] herbicides (10, 21). Photosynthetic electron transport (the site of action of at least 50% of all known herbicides), apparently is not involved in herbicidal activity of acifluorfen in higher plants (7, 10, 21, 28, 32, but compare 3). The photoreceptor appears to be a carotenoid. Etiolated tissue (25) and yellow plants derived from genetic mutations (21) or treatment with tentoxin (8) are as sensitive to acifluorfen as is green tissue. Albino mutants (10, 21) and white plants derived from the application of herbicides that block carotenoid biosynthesis (8, 26), however, are virtually insensitive to DPEs. The development of herbicidal damage upon exposure of acifluorfen-treated plants to light is manifested, after a short lag period, in a simultaneous loss in selective permeability of the plasmalemma (8, 14, 25, 26, 32), production of lipid hydroperoxides (3, 14, 29), and evolution of both ethane and ethylene (3, 14, 15, 29). Damage at the ultrastructural level is seen first as disruption of the plasmamembrane, tonoplast, and chloroplast envelope, followed by general disintegration of cytoplasmic organelles and, ultimately, the thylakoids (14, 25, 26).

Oxygen is required for herbicidal injury; both a nitrogen atmosphere and antioxidants diminish the effects of DPEs (16, 26, 29). The involvement of light and O_2 and the production of lipid hydroperoxides has led to the proposal that DPEs form toxic oxygen radicals which initiate free radical chain reactions in cell membranes (3, 26). Alternatively, the DPEs may act by prohibiting the quenching of free radicals that may be produced in the light in the absence of the herbicide. The latter hypothesis is supported by the finding that ascorbate levels decrease in mustard seedlings treated with oxyfluorfen (17).

Ascorbate and GSH can inactivate a variety of toxic oxygen radicals and are considered the first line of defense against oxidative stress in plants (19). The purpose of the present investigation was to examine the effect of acifluorfen on ascorbate and glutathione levels in herbicide-treated tissue and the enzymes that modulate the levels of these endogenous reductants. Both glutathione and ascorbate were rapidly depleted in the light along with the activity of enzymes responsible for maintaining their redox states. The rapid destruction of GSH and ascorbate is indicative of the severe oxidative stress occurring in plants exposed to DPEs.

MATERIALS AND METHODS

Plant Material. Cucumber seeds (*Cucumis sativus* cv 'Straight Eight', Hollar and Co., Inc.[3] Rocky Ford, CO) were sown in flats containing Jiffy Mix (JPA, West Chicago, IL) under constant fluence rate of 150 μmol m^{-2} s^{-1} of white light. Cotyledons from 5- to 7-d-old seedlings were used for all experiments. FR grown plants were grown as above except under constant FR illumination as previously described (7). Under these FR light conditions the plant material was pale-green in appearance.

Herbicide Treatments. Cotyledon discs (4 mm diameter) were cut from the margins of the cotyledons, avoiding the midrib, with a brass cork borer. The discs were rinsed in water and

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[2] Abbreviations: DPE, diphenyl ether; DHA, dehydroascorbate; SOD, superoxide dismutase; FR, far red.

[3] Mention of a trademark, proprietary product or vendor does not constitute a guarantee of warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other vendors that may also be suitable.

floated on 0.1 mM Mes-NaOH (pH 6.5), 0.1% (w/v) sucrose in the presence or absence of 30 μM Na-acifluorfen. One hundred to 200 discs per time point were floated on 15 ml of medium in 9 cm diameter Petri dishes. Discs were preincubated for 20 h in the dark, 25°C to allow adequate penetration of herbicide. Petri dishes were exposed to light in a growth chamber at 25°C under a white light fluence rate of 450 μmol m^{-2} s^{-1} provided by mixed 100 W incadescent, 135 W low-pressure sodium, and 40 W cool white fluorescent lamps. Conductivity of the treatment solution was assayed with a conductivity meter (model 4503A, Amber Science), having the capacity to assay and return 1 ml of solution to the Petri dish.

Ascorbate, Dehydroascorbate, and Glutathione Determinations. Leaf discs (100–200) were frozen in liquid N_2 and then extracted and assayed for ascorbate and DHA essentially as described by Foyer *et al.* (12).

Glutathione was extracted by grinding discs in 5% w/v sulfosalicylic acid and centrifuging the extract at 7000*g* for 10 min. Both GSSG and GSH were assayed in the supernatant by a modification of the procedure of Griffith (13) as described by Law et al. (19).

Enzyme Assays. Enzymes were extracted by grinding 200, 4 mm diameter discs in 7 ml 50 mM KH_2PO_4 (pH 7.0) at 4°C. A 7000*g* × 10 min supernatant was used for all assays. Enzymic activity was determined by previously published procedures as follows: DHA reductase (23), GSSG reductase (30), ascorbate free radical reductase (1), catalase (2), Cyt *c* oxidase (31), glucose-6-P dehydrogenase (31), NAD-malate dehydrogenase (31), duroquinone NADH oxidase (6), glyceraldehyde-3-P dehydrogenase (18), NADH Cyt *c* reductase (31), peroxidase (2), ascorbate oxidase (24), SOD (22), lipoxygenase (27), and acid phosphatase (4). Enzyme activity of treated tissue is expressed as a percentage of untreated tissue activity at the same point in time.

Chemicals. All biochemical reagents were of the highest quality available. Technical grade (>99%) acifluorfen was a generous gift of the Rohm and Haas Corporation, Spring House, PA. Dehydroascorbate was purchased from Fluka Fine Chemicals (Basel, Switzerland).

RESULTS

Electrolyte Leakage. For comparison purposes, electrolyte leakage was assayed as a rapid measure of herbicidal damage. We previously found electrolyte leakage to be one of the earliest effects of acifluorfen (14). Acifluorfen-caused electrolyte leakage was detectable after 1 h of light and increased linearly from this time through 5 h of light (Fig. 1).

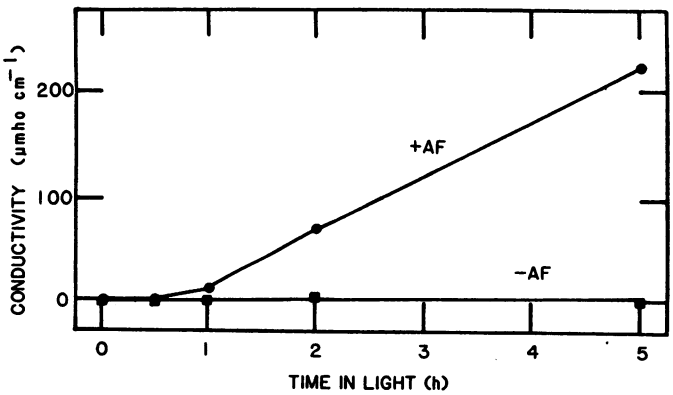

FIG. 1. Effect of acifluorfen on electrolyte leakage from cucumber cotyledons discs during exposure to white light (450 μE m^{-2} s^{-1}) after 20 h of dark incubation with or without the herbicide. Values are the increase in conductivity of the bathing media after initial exposure to light.

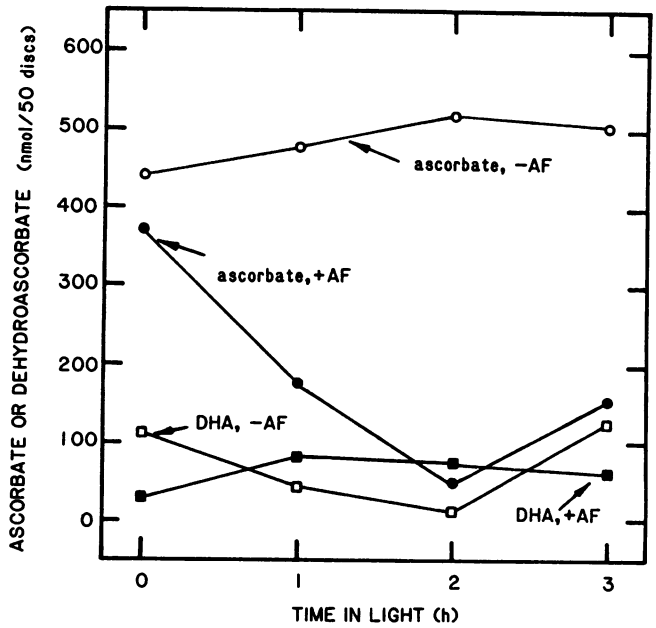

FIG. 2. Effect of acifluorfen on the ascorbate and dehydroascorbate content of green cucumber cotyledons. The results are from a typical experiment; however, all assays were triplicated and experiments were either duplicated or triplicated.

Ascorbate/Dehydroascorbate. Acifluorfen caused a dramatic depletion of ascorbate in herbicide treated cucumber cotyledons upon exposure to light (Fig. 2). In five replicate experiments it was noted consistently that the initial ascorbate concentration in acifluorfen-treated cotyledon tissue was 16% lower than the control after 20 h of dark pretreatment. After 1 h of light, ascorbate in acifluorfen treated discs actually diminished by 40% but as a percentage of ascorbate in the control tissue, it decreased approximately 58% because ascorbate began to accumulate in control tissue in the light. After 3 h of exposure to light only one-third as much ascorbate remained in herbicide-treated tissue. The effects on ascorbate content were more rapid and of relatively greater magnitude than effects on cellular leakage (Fig. 1).

In no experiment did the amount of DHA in herbicide-treated discs increase in proportion to the decrease seen in ascorbate (Fig. 2). The levels of DHA in both control and treated tissue consistently were similar, even after 3 h of illumination.

Photosynthetic electron transport has been implicated in the mechanism of action of acifluorfen (3). It was of interest, therefore, to determine the behavior of ascorbate in photosynthetically incompetent cucumber tissue exposed to acifluorfen. FR light grown cucumber plants have 30% of the Chl and 50% of the carotenoids of white-light grown plants and are incapable of full chain photosynthetic electron transport and CO_2-dependent O_2 evolution (data not shown). Ascorbate content of FR light grown plants was very sensitive to acifluorfen, decreasing by nearly 90% within 1 h of being placed in white light (Fig. 3). Again, DHA levels did not increase in proportion to the decreases in ascorbate during the time course.

Glutathione. The effect of acifluorfen on the reduced glutathione content of cucumber cotyledons was similar to that observed with ascorbate (Fig. 4). The amount of GSH diminished by 40% during the 1st h of light and continued to decrease. In contrast, reduced glutathione levels in control tissue increased in the first few hours of illumination (Fig. 4). The impact of the herbicide on the concentration of GSH in cucumber is seen by the large drop in GSH calculated as percentage of control. GSH content in acifluorfen-treated tissue fell approximately 50% in the first 60 min of light (Fig. 4). The effects of acifluorfen on GSH

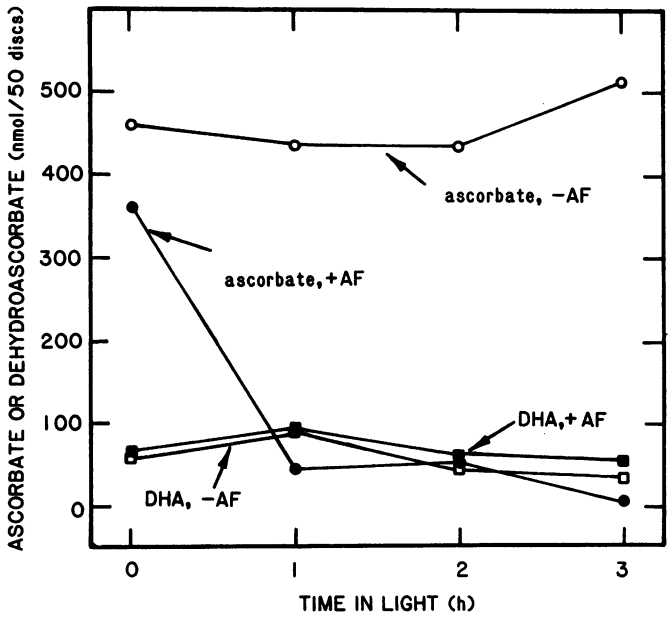

FIG. 3. Effect of acifluorfen on the ascorbate and dehydroascorbate content of cucumber cotyldeons from plants grown under FR light. Plants were grown under constant FR illumination for 5 to 7 d. Cotyledon discs were then cut under green light and incubated for 20 h in darkness with or without acifluorfen before exposure to white light (450 μmol m^{-2} s^{-1}).

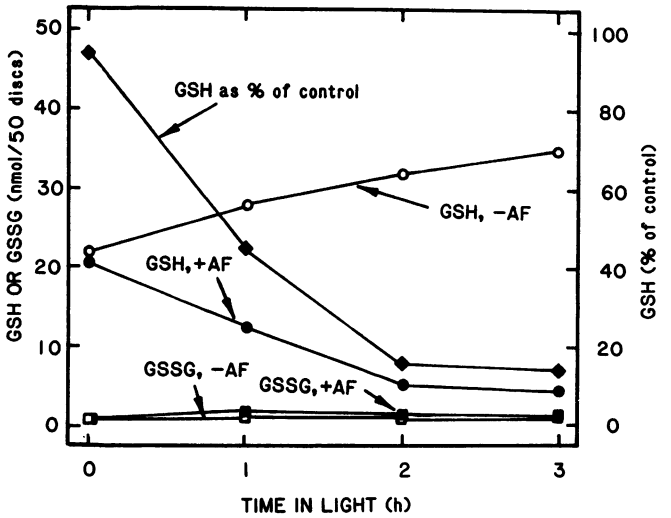

FIG. 4. Effect of acifluorfen on the levels of reduced and oxidized glutathione in green cucumber cotyledons.

content were more rapid and of greater magnitude than effects on electrolyte leakage (Fig. 1). Similar to what was observed with DHA, GSSG levels remained low and nearly constant for both control and herbicide-treated tissue (Fig. 4).

Protective Enzymes. The depletion of both ascorbate and GSH in the light suggested that acifluorfen may prevent the reduction of both DHA and GSSG. To examine this possibility, the *in vitro* and *in vivo* effects of the herbicide on the activities of a number of enzymes involved in the maintenance of the oxidation/reduction state of these reductants were determined. Acifluorfen had no *in vitro* effect on the activity of any of the enzymes assayed in control tissue, even at levels that were 10 times higher than those required for a threshold herbicide response in intact tissues (data not shown). When acifluorfen-treated cucumber cotyledon discs were exposed to light, however, the activities of extracted

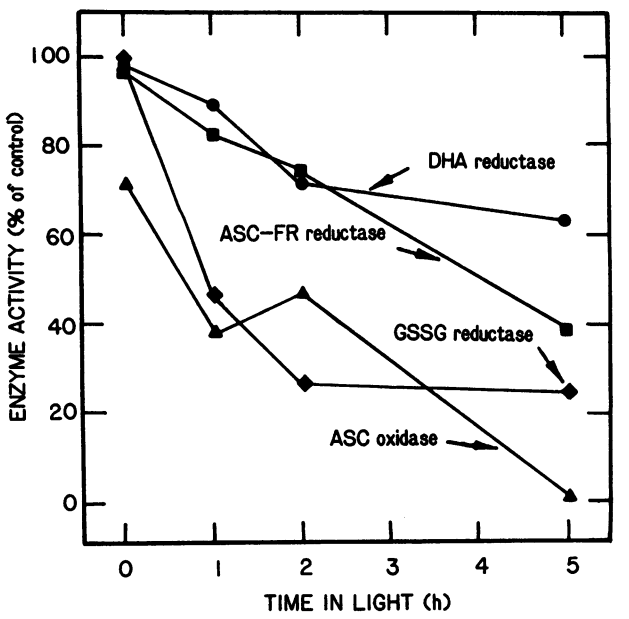

FIG. 5. Effect of acifluorfen on enzymes that affect the ratio of oxidized to reduced ascorbate or glutathione. The average (±1 SE) enzyme activities (product formed or substrate degraded) in 1 ml of control (untreated) extract during the 5 h time course were: DHA reductase, 14.6 ± 0.5 μmol min^{-1}; ascorbate-free radical reductase, 15.0 ± 0.13 μmol min^{-1}; glutathione reductase, 2.23 ± 0.34 μmol min^{-1}; and ascorbate oxidase, 49.0 ± 4.9 μmol min^{-1}. Activities of the enzymes from acifluorfen-treated tissues are presented as a percentage of activities from control tissues at the same points in time.

GSSG reductase, DHA reductase, ascorbate oxidase, and ascorbate free-radical reductase, diminished (Fig. 5). The most sensitive enzymes were GSSG reductase and ascorbate oxidase, which retained less than 50% of the activity found in the untreated control after 1 h of light. A consistent observation in replicate experiments was a decrease in recoverable ascorbate oxidase activity after dark preincubation. This decrease continued more rapidly in the light (Fig. 5).

Acifluorfen strongly affected other enzymes which are directly or indirectly involved in protective mechanisms. The activity of SOD, catalase, and peroxidase decreased rapidly in the light (Fig. 6A). Peroxidase consistently had lower activity in acifluorfen-treated tissue after the 20 h dark preincubation in replicate experiments. A number of other enzyme activities associated with various cytoplasmic compartments were determined in order to assess in a general way the photooxidative damage to enzymes caused by acifluorfen. Enzyme activities associated with vacuoles (acid phosphatase), mitochondria (malate DH and Cyt *c* oxidase), cytoplasm (malate DH and glucose-6-P DH), chloroplasts (malate DH and glyceraldehyde-3-P DH), plasmamembrane (duroquinone NADH oxidase), and ER (NADH Cyt *c* reductase and acid phosphatase) show a similar pattern of inactivation (Fig. 6B). NADH Cyt *c* reductase was particularly sensitive to acifluorfen. Lipoxygenase which oxidizes linoleic acid to form linoleic hydroperoxides, in contrast to all other enzymes, consistently increased in activity relative to the control from 0 to 2 h of light (Fig. 6B). Although lipoxygenase activity as a percentage of control appears to decline between 2 and 5 h, there was no reduction in the absolute level of lipoxygenase activity in the treated tissue during the 5 h time course, since the activity of the control increased about 2-fold during this time.

DISCUSSION

Acifluorfen causes the rapid depletion of endogenous ascorbate in the light (Figs. 2 and 3). Similarly, Kunert (17) observed a

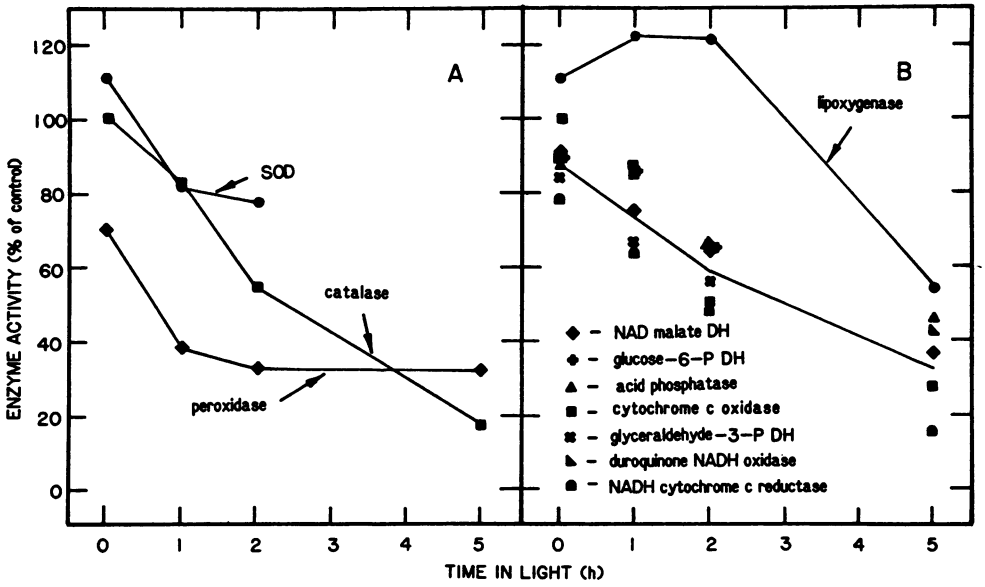

FIG. 6. Effects of acifluorfen on (A) protective enzymes and (B) enzymes associated with various organelles in green cucumber cotyledons. In part B, a generalized curve is drawn for all enzymes except lipoxygenase. The average (±1 SE) enzyme activities (standard units or substrate degraded or product formed) in 1 ml of control (from untreated discs) extract during the time course were: SOD, 126 ± 3 units; catalase, 271 ± 9 μmol min^{-1}; peroxidase, A_{470} 33.5 ± 1.6 min^{-1}; NAD malate dehydrogenase, 14.6 ± 0.53 μmol min^{-1}; glucose-6-P dehydrogenase, 159 ± 5 nmol min^{-1}; acid phosphatase, 176 ± 9 μmol min^{-1}; Cyt *c* oxidase, 5.06 ± 0.49 μmol min^{-1}; glyceraldehyde-3-P dehydrogenase, 1.70 ± 0.09 μmol min^{-1}; duroquinone NADH oxidase, 2.28 ± 0.28 μmol min^{-1}; NADH Cyt *c* reductase, 53.6 ± 5.8 μmol min^{-1}. Lipoxygenase activity increased in the control during the time course from 0.92 ± 0.03 μmol min^{-1} at 0 h to 1.92 ± 0.12 μmol min^{-1} at 5 h. Activities of the enzymes from treated tissues are expressed as a percentage of the control tissue activities at the same points in time.

decrease in ascorbate in mustard seedlings, but not until 24 h after exposure to the DPE oxyfluorfen. Ascorbate destruction can occur independently of photosynthetic electron transport (Fig. 3). This finding is in general agreement with previous observations that photosynthetic electron transport inhibitors do not affect DPE-induced damage to higher plants (8, 21, 26, 28). The absence of an accumulation of DHA may be explained by its facile degradation to oxalic and L-threonic acids which has been observed previously in paraquat-treated chloroplasts (12, 19).

The 50% decrease in ascorbate within 1 h indicates that the oxidation of ascorbate is one of the earliest effects found in acifluorfen-treated plants (Figs. 2, 3; [14]). Ascorbate and GSH are the major components in the ascorbate-GSH cycle occurring in the chloroplast (12, 19). The depletion of both of these reductants explains why CO_2-dependent O_2 evolution is one of the most rapidly affected metabolic processes when herbicide-treated plants are placed in the light (14).

Finckh and Kunert (11) have recently found that the ratio of ascorbate to α-tocopherol in plant tissues is more important than absolute levels of each protectant in affording protection from photooxidative damage due to oxyfluorfen treatment, although there was a positive correlation between ascorbate concentration and protection from the herbicide. Ascorbate can apparently regenerate α-tocopherol from α-tocopherol radicals (20); however, high levels of ascorbate in the presence of metal ions can stimulate lipid peroxidation (9). Thus, changes in ascorbate levels can influence free radical-caused damage by several mechanisms.

The depletion of GSH is similar to ascorbate and represents an early effect of acifluorfen damage (Fig. 4). Glutathione may be oxidized either by directly quenching oxy-radicals, reducing ascorbate, or reducing labile sulfhydryl groups on proteins (12, 19, 33). The oxidation of GSH to a thiyl radical or glutathione sulfonate may be responsible for the lack of accumulation of GSSG (Fig. 4).

The reduction in extractable activity of DHA reductase and ascorbate free-radical reductase alone cannot account for the depletion in endogenous ascorbate since ascorbate falls by 50% in 1 h while these enzyme activities fall only 8 and 13%, respectively (Figs. 2, 3, and 5).Glutathione reductase activity, however, drops 55% in 1h, more than the level of GSH (Figs. 4 and 5). Ascorbate oxidase, an enzyme of unknown function which occurs in large amounts in the *Cucurbitaceae*, was assayed with the expectation that its activity may be inversely related to ascorbate depletion. Ascorbate oxidase activity in acifluorfen-treated cucumber was consistently lower than control in the dark and dropped off rapidly in the light (Fig. 5). The reasons for this pattern were unclear.

The protective enzymes catalase, peroxidase, and SOD and enzymes associated with various organelles follow a pattern which indicates general cellular disruption (Fig. 6A). Peroxidase seems to be particularly sensitive to acifluorfen. This is not surprising, because peroxidase is localized in the vacuole and near the plasmalemma and both the tonoplast and plasmalemma have been shown to be early sites of acifluorfen-caused damage (14). Ozone causes leakage of peroxidase from cell walls (5). Although the oxidative damage of acifluorfen resembles the effects of ozone, it is unlikely that the diminution of enzyme activity at early time points is a result of the wholesale leakage of cellular contents. Acifluorfen causes leakage of electrolytes and sugars from treated plants (8, 14, 25, 26; Fig. 1); however, protein and enzyme activity (acid phosphatase, ascorbate oxidase, and peroxidase) were undetectable in the medium until after 5 h of exposure to light (data not shown). Also, the onset of loss of enzyme activities was more rapid than loss of electrolytes.

It is interesting to note that lipoxygenase activity consistently was stimulated in darkness by acifluorfen (Fig. 6A). Acifluorfen causes membrane leakage during the 20 h dark incubation as seen by slight increases in conductivity and decreases in fresh weight relative to the control (data not shown). The release of polyunsaturated fatty acids (*e.g.* linolenic) to a small degree in darkness and to a larger extent in the light may account for the increases in lipoxygenase seen in herbicide-treated tissue. The

activity of lipoxygenase is significant in regard to the mechanism of action of the DPEs. Lipoxygenase produces fatty acid hydroperoxides which can destroy the selective permeability of cellular membranes and result in disorganization of cellular compartmentation. In addition, a well-known side reaction of lipoxygenase is the co-oxidation of carotenoids. Carotenoids have been implicated in the mechanism of action of acifluorfen (3, 8, 10, 21) and appear to be among the pigments most sensitive to photo-oxidative destruction by acifluorfen (15), although substantial loss of carotenoids in acifluorfen-treated tissue does not occur for several hours in white light (14).

The present study supports previous work on the mechanism of action of the DPE herbicides indicating that DPE-treated plants are under severe photo-oxidative stress. This oxidative stress is manifested in a rapid depletion of the two major cellular antioxidants and the irreversible loss of activity of a large number of enzymes.

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