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Received for review September 7, 1982. Revised manuscript received December 20, 1982. Accepted March 7, 1983. Presented at the Pesticide Division, 184th National Meeting of the American Chemical Society, Kansas City, MO, Sept 16, 1982.

Acifluorfen Increases the Leaf Content of Phytoalexins and Stress Metabolites in Several Crops

Tâmas Kömives[1] and John E. Casida*

Leaves treated with acifluorfen [sodium 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate] contain greatly increased levels of *N*-feruloyl-3-methoxytyramine (spinach) and of 10 phytoalexins, i.e., glyceollins I, II, and III and glyceofuran (soybean), phaseollin (bean and pinto bean), pisatin (pea), medicarpin and wyerone (broad bean), xanthotoxin (celery), and hemigossypol (cotton). Enhanced synthesis of these compounds is related to the acifluorfen concentration and exposure time to light. The phytotoxicity of acifluorfen and oxyfluorfen [2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene] to spinach is counteracted by appropriate treatments with (aminooxy)acetic acid, L-2-(aminooxy)-3-phenylpropionic acid, or silver nitrate and by heat shock. Under certain conditions soybean injury is ameliorated by combining (aminooxy)acetic acid with acifluorfen and silver nitrate with oxyfluorfen. These relationships for diphenyl ether (DPE) herbicides and protective treatments resemble those for other stress factors with associated increases in lipid peroxidation, membrane permeability, ethylene production, and phenylalanine ammonia-lyase (PAL) activity. Although increased PAL activity is not a primary lesion, it may play an important role in DPE herbicide action.

Herbicide stress may lead to accumulation of secondary plant products: e.g., diphenyl ethers (DPEs) increase the *N*-feruloyl-3-methoxytyramine (FMT) content of spinach leaves (Kömives and Casida, 1982; Suzuki et al., 1981), 2,4-D and maleic hydrazide promote accumulation of scopolin and scopoletin in tobacco (Wender, 1970), and atrazine, bentazon, endothall, and oxadiazon increase the level of certain isoflavonoids in soybeans and navy beans (Rubin et al., 1979a,b). Some of the same secondary compounds accumulate in response to other abiotic elicitors (e.g., heavy metals, surfactants, and organic solvents) and to microorganisms (i.e., phytoalexins are produced as natural self-defense chemicals) (Bailey, 1982). Formation of these secondary products, stress metabolites, or phytoalexins is usually initiated by or associated with cell death caused by the chemical or microorganism (Bailey, 1982).

Acifluorfen [sodium 5-[2-chloro-4-(trifluoromethyl)-phenoxy]-2-nitrobenzoate], oxyfluorfen [2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene], and other DPE herbicides are of particular interest since their phytotoxic action in many plants is accompanied by strong induction of a key enzyme in biosynthesis of several phytoalexins, i.e., phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) (Kömives and Casida, 1982). Thus, preliminary studies indicate that acifluorfen treatment of beans and soybeans, as well as spinach, strongly incrases their levels of specific organosoluble phenolics and that these phenolics may include isoflavonoid phytoalexins (Kömives and Casida, 1982).

This study examines a variety of acifluorfen-treated plants for possible changes in their secondary products, with emphasis on crops with well-characterized but structurally divergent phytoalexins (Figure 1). It also

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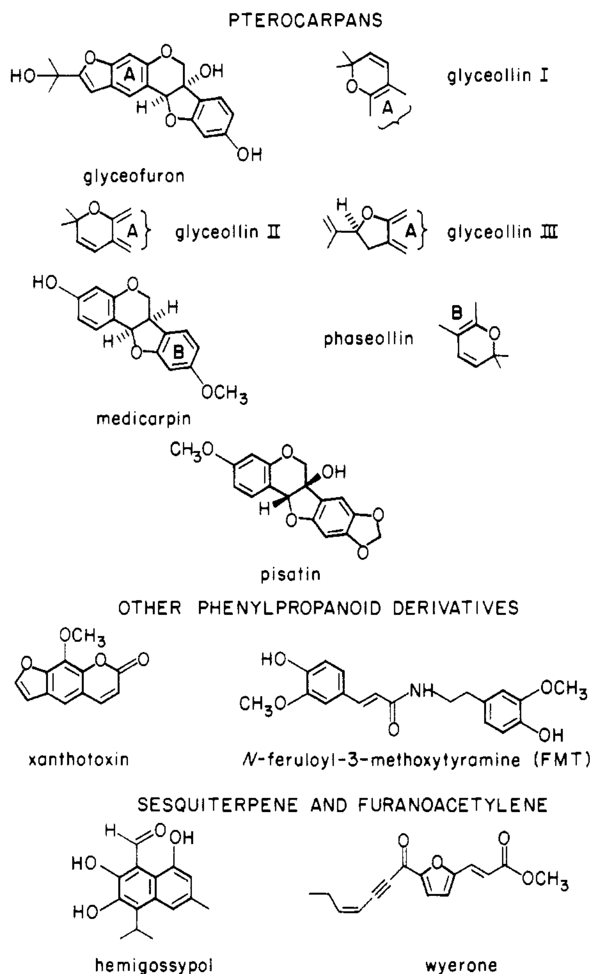

Figure 1. Structures of 11 secondary plant metabolites increased in concentration by acifluorfen.

attempts to counteract the DPE-type phytotoxicity.

MATERIALS AND METHODS

Chemicals. Compounds provided as gifts were as follows: acifluorfen (pure) and oxyfluorfen (formulated Goal herbicide) (Rohm and Haas Co., Philadelphia PA); glyceollins I, II, and III (N. T. Keen, University of California, Riverside, CA); phaseollin (O. I. Huisman, University of California, Berkeley, CA); L-2-(aminooxy)-3-phenylpropionic acid (AOPP) (N. Amrhein, Ruhr-Universität, Bochum, West Germany, and B. Fritig, Institut de Biologie Moléculaire et Cellulaire de CNRS, Strasbourg, France); L-2-amino-4-(2-aminoethoxy)-*trans*-3-butenoic acid or (aminoethoxy)vinylglycine (AVG, Hoffman-La Roche, Nutley, NJ). FMT was synthesized in this laboratory (Suzuki et al., 1981). (Aminooxy)acetic acid (AOA), 1-aminocyclopropane-1-carboxylic acid (ACC), poly(oxyethylene) sorbitan monolaurate (Tween-20), and other compounds not indicated above were from commercial sources.

Plant Materials and Treatments. Individual fresh leaves of bean, cotton, pinto bean, spinach, and tomato (*Lycopersicon esculentum* Mill.) and cut seedlings of broad bean, celery, pea, and soybean were preconditioned by placing the stem in 5 mL of water for 4 h. Acifluorfen (1 ppm = 2.6 μM), AOA (1 ppm = 11 μM), AOPP (1 ppm = 5.5 μM), and AVG (1 ppm = 6.3 μM) were administered as before (Suzuki et al., 1981) by placing the stem in 20-mL solutions, whereas oxyfluorfen (1 ppm = 2.8 μM) and $AgNO_3$ (1 ppm = 5.9 μM; in 0.05% Tween-20) were applied to the surface by immersing the leaves for 20 s in aqueous solutions and then holding them with the stem in 20 mL of water. Heat shock (Heath, 1979) involved dipping spinach leaves into distilled water at up to 60 °C for 25 s and then placing the stem in 20 mL of water. Unless specified otherwise, all plant material was held in the light under normal laboratory conditions.

Spinach leaf disks (19-mm diameter) were preconditioned by holding them overnight on moist filter paper in the light. Three treatments or combinations thereof and the relevant controls were examined: 1-min dip in acifluorfen (500 ppm) or oxyfluorfen (100 ppm) aqueous solution; 30-min floating on AVG (20 ppm) aqueous solution; water or 0.05% Tween-20 as controls. For ethylene and ethane determinations, three aged and treated disks were placed in a 10-mL glass vial which was sealed with a rubber septum and held at 25 °C in the light. The vials were flushed with air every 8 h in the overall experiment of 72-h duration.

Analyses. Literature methods involving thin-layer chromatography (TLC) followed by UV or visible spectrophotometry were used to quantitate the glyceollins and glyceofuran in soybeans (Ingham et al., 1981), phaseollin in bean and pinto bean (Cruickshank and Perrin, 1971), pisatin in pea (Banks and Dewick, 1982), wyerone and medicarpin in broad bean (Robeson, 1978), xanthotoxin in celery (Johnson et al., 1973), hemigossypol in cotton (Stipanovic et al., 1975), and FMT in spinach (Kömives and Casida, 1982; Suzuki et al., 1981). Each compound was purified by TLC until it appeared as a single spot under UV (254 or 366 nm). The secondary metabolites were identified by cochromatography with standards (when available), UV spectra in neutral, basic (for phenols), and acidic (for pisatin) ethanol solutions, and chemical ionization mass spectrometry (CI-MS, methane at 0.8 torr) or electron impact mass spectrometry (EI-MS, 70 eV). Their TLC and MS characteristics and the UV absorption data used for quantitation are given in Table I. The UV and MS comparisons were made with authentic standards of glyceollins I, II, and III, phaseollin, xanthotoxin, and FMT and with literature values in other cases. Isomer ratios for the glyceollins were determined by high-pressure liquid chromatography (HPLC) (Waters μPorasil column. 7.8 mm i.d. × 30 cm, eluted with hexane–EtOAc, 2:1, at 3 mL/min, 254-nm detector; elution times of 14.7, 15.7, and 17.9 min for glyceollins I, II, and III, respectively).

Ethylene and ethane were analyzed in the headspace of the leaf disk experiments by gas–liquid chromatography (Sandmann and Böger, 1982b) with retention times of 2.0 and 2.7 min, respectively, at 105 °C isothermal. Malondialdehyde was determined by extraction with 0.1% trichloroacetic acid followed by the thiobarbituric acid reaction (Dhindsa et al., 1981). Total phenols in a 50% methanol extract of the leaves were determined by using the Folin reagent (Forrest and Bendall, 1969). PAL was analyzed by the UV method (Kömives and Casida, 1982). Antifungal activity was assayed by the in situ TLC technique with *Cladosporium cucumerinum* (Keen et al., 1971).

RESULTS

Phytotoxicity of Acifluorfen in Eight Crops. Light is required for acifluorfen-treated plants to develop phytotoxic signs (Table II). The sensitivity order for the plants under the conditions examined was broad bean and pea > bean, cotton, pinto bean, and spinach > soybean > celery. The severity of phytotoxicity increased at higher doses and longer treatment times, with death requiring at least 2–5 days. The sequential symptoms of the leaves were appearance of water-soaked spots, color change to yellow, brown, or black (the latter for broad bean), wilting

Table I. Plant Origin and Properties of Eleven Secondary Metabolites Increased in Concentration by Acifluorfen

compound	plant	TLC[a] solvent[b] (R_f)	UV absorption[c] λ_{max}	UV absorption[c] ϵ	CI-MS, m/e (rel intensity) $(M + 1)^+$	CI-MS, m/e (rel intensity) base peak
glyceollins I, II, and III[d]	*Glycine max* L. soybean	B (0.48), D (0.46), E (0.50)	285	10 300	338 (32)[e]	305
glyceofuran	*Glycine max* L. soybean	B (0.16), E (0.27), I (0.35)	293	10 000	354 (8)[e]	318
phaseollin	*Phaseolus vulgaris* L. bean	B (0.81), C (0.74), I (0.68)	280	9 800	323 (75)	165
pisatin	*Pisum sativum* L. pea	B (0.39), C (0.74), G (0.71)	307	7 200	315 (87)	177
medicarpin	*Vicia faba* L. broad bean	B (0.45), C (0.46), D (0.21)	287	7 900	271 (100)	
wyerone	*Vicia faba* L. broad bean	A (0.61), D (0.58), J (0.49)	350	27 000	259 (100)	
xanthotoxin	*Apium graveolens* L. celery	D (0.29), F (0.42), H (0.38)	250	30 300	217 (100)	
hemigossypol	*Gossypium hirsutum* L. cotton	K (0.12), L (0.36)	553[f]	21 500[f]	261 (100)	
FMT	*Spinacia oleracea* L. spinach	B (0.39), C (0.51), D (0.22)	323	21 000	343 (57)[e]	150

[a] Silica gel 60 chromatoplates (Merck, Darmstadt, West Germany, F254, 0.25 mm thick) except hemigossypol on polyamide 11 chromatoplates (Macherey, Nagel & Co., Düren, West Germany, F254, 0.2 mm thick). [b] Solvent systems: A, $CHCl_3$-MeOH (50:1); B, $CHCl_3$-MeOH (19:1); C, $CHCl_3$-EtOAc-AcOH (8:1:1); D, hexane-acetone (3:1); E, acetone-$CHCl_3$-concentrated NH_4OH (50:50:1); F, CH_2Cl_2-EtOH (70:1); G, toluene-EtOAc (8:1); H, hexane-EtOAc (1:1); I, toluene-acetone-$CHCl_3$ (8:7:5); J, ether-hexane (3:1); K, benzene-$CHCl_3$-MeOH-AcOH (150:50:3:2); L, $CHCl_3$-acetone-HCO_2H (95:4:1). [c] In EtOH except wyerone and FMT in MeOH. Data from text references or as follows: Lazarovits and Ward (1982) for glyceollins; Smith et al. (1971) for medicarpin; Mansfield (1982) for wyerone; Scheel et al. (1963) for xanthotoxin. [d] Individual glyceollin isomers are readily separated by HPLC (see Materials and Methods). [e] EI-MS, $M^{+\cdot}$. [f] Phloroglucinol derivative (Stipanovic et al., 1975).

Table II. Sensitivity of Eight Crops to Acifluorfen and Increase in Concentration of Secondary Metabolites

plant	acifluorfen, ppm[a] minimum toxic dose	acifluorfen, ppm[a] dose used to increase sec metab	secondary plant metabolite: compound	ppm[b] control	ppm[b] treated
soybean	1	5	glyceollins	<1	79 ± 17
			glyceofuran	<1	16 ± 5
bean[c]	0.2	5	phaseollin	<1	47 ± 10
pea	0.04	5	pisatin	7 ± 2	133 ± 14
broad bean	0.03	5	medicarpin	<1	45 ± 7
			wyerone	<1	15 ± 4
celery	5	50	xanthotoxin	6 ± 2	24 ± 4
cotton	0.2	5	hemigossypol	<1	59 ± 15
spinach	0.2	5	FMT	<0.5	17 ± 5

[a] A 72-h exposure. Normal laboratory lighting was used. No phytotoxic signs evident in each case at 5 ppm for 5 days in the dark. [b] Average and SD at 72 h in three experiments. The treated values were essentially the same as those of the control for plants held in the dark. [c] Similar findings for pinto bean.

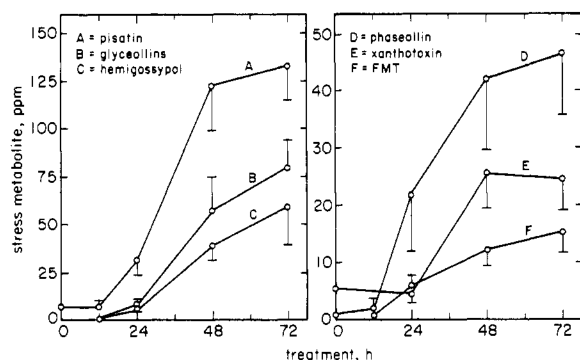

Figure 2. Leaf content of six secondary plant metabolites at various treatment times with acifluorfen at 5 or 50 ppm (celery for xanthotoxin only). Data for FMT are from Kömives and Casida (1982). Species involved are indicated in Table II.

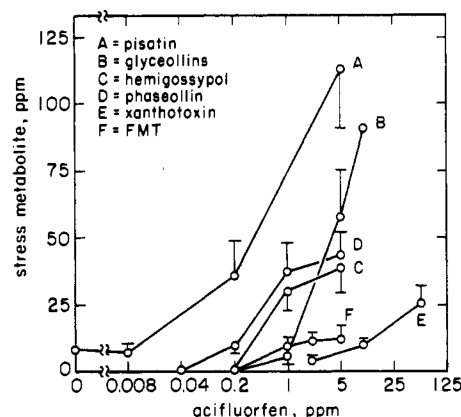

Figure 3. Leaf content of six secondary plant metabolites at various treatment levels of acifluorfen with a 48-h exposure time. Data for FMT are from Kömives and Casida (1982). Species involved are indicated in Table II.

and desiccation first evident at 24 h, and chlorosis, usually after the appearance of necrotic lesions. Red-brown pigments appeared in the veins and as small spots in acifluorfen-treated cotton leaves.

Secondary Metabolites Including Antifungal Compounds Produced in Eleven Acifluorfen-Treated Crops and Weeds. Acifluorfen has little or no effect within 72 h on the total phenolic content of acifluorfen-treated leaves of bean, broad bean, celery, cotton, pea, pinto bean, soybean, spinach, and tomato when using phytoxic doses (5 ppm except 50 ppm for celery), i.e., an overall average ± SD for the nine plants of 107 ± 28% for the treated vs. the controls. However, acifluorfen greatly alters the level of selected components of each crop examined.

Eleven secondary metabolites (Figure 1) were considered in more detail (Figures 2 and 3; Table II). Their leaf content was increased by 4-fold for xanthotoxin in celery

Table III. Effects of Two Ethylene Precursors, Glyphosate, and Various Toxicants on Spinach Leaves and Their Content of *N*-Feruloyl-3-methoxytyramine

compound[a]	ppm	injury[b]	FMT, ppm[c]
control		–	<0.5
ethephon	20	+	1.7 ± 0.8
ACC	60	++	6.3 ± 2.0
glyphosate	50[d]	+++	<0.5

[a] Other compounds increasing FMT content at toxic levels were (ppm, stem uptake for 48 h): DNA intercalating agents ethidium bromide (60) and 9-aminoacridine (60); inhibitors of protein and nucleic acid synthesis cycloheximide (2) and 6-methylpurine (60); alkylating agents (60 ppm each) iodoacetic acid, iodoacetamide, and *N*-ethylmaleimide. [b] Injury rated as none (–) to severe (+++) in at least five experiments. [c] Average and SD at 48 h in three experiments. [d] 96 h used to allow development of phytotoxic signs.

to >79-fold for the glyceollins in soybean. The enhanced biosynthesis shows appropriate dependency on exposure time (Figure 2), acifluorfen dose (Figure 3), and light (Table II). The ratio for glyceollins I, II, and III was 1:7:11 with acifluorfen in leaves as compared to 1:3:6 in soybean plants induced with iodoacetic acid (Ingham et al., 1981), 2.3:1:2.4 in cotyledons treated with $CuCl_2$ (Lyne et al., 1976), and 8:1:1 in fungus-infected hypocotyls (Moesta and Grisebach, 1981).

The induction of stress metabolites by acifluorfen is not restricted to either the compounds or the plants listed in Table I. Several UV-quenching (254 nm) bands, in addition to those in Table I, appeared on TLC of extracts of acifluorfen-treated but not the control bean, pinto bean, soybean, pigweed (*Amaranthus retroflexus* L.), and lambsquarters (*Chenopodium album* L.). In situ TLC bioassays of extracts of acifluorfen-treated plants revealed one antifungal compound with tomato and two with cotton (in addition to hemigossypol). The tomato product was neither UV quenching nor fluoresent at 254 or 366 nm (silica gel 60, F254, 0.25 mm thick, cyclohexane–EtOAc, 3:1, R_f 0.43), while the two cotton compounds gave a yellow fluorescence at 366 nm (solvent system L, R_f 0.18 and 0.44). These unidentified compounds in acifluorfen-treated bean, pinto bean, and soybean may be phenylpropanoid derivatives (Ingham, 1982) while the antifungal products in cotton and tomato may be terpenoids (Essenberg et al., 1982; Kuč, 1982). The spinach phenylpropanoid, FMT, was not fungicidal at 500 μg/spot in the TLC bioassay.

Increased levels of secondary metabolites are evident in each case (Figure 2) before the appearance of necrotic lesions, although a phytotoxic dose was always required to induce high levels of secondary metabolites.

Injury and FMT Levels with Other Toxicants in Spinach. The association of leaf injury with increased FMT levels noted for acifluorfen (Table II) occurs also with many but not all other toxicants (Table III) (Suzuki et al., 1981). The ethylene precursors ethephon and ACC increase the FMT content as does a variety of DNA intercalating agents, inhibitors of protein and nucleic acid synthesis, and alkylating agents. Glyphosate injury without increased FMT content may be due to inhibiting the biosynthesis of phenylalanine and tyrosine (Steinrücken and Amrhein, 1980), the precursors of FMT (Kömives and Casida, 1982).

Effects of Acifluorfen and Oxyfluorfen on Spinach Leaf Disks. Preliminary experiments indicate that phytotoxic doses of acifluorfen and oxyfluorfen increase by more than 10-fold the levels of ethylene and ethane evolved and the leaf content of FMT and increase by more than 4-fold the PAL activity and malondialdehyde content.

Table IV. Treatments Counteracting the Phytotoxic Effects of Acifluorfen (5 ppm, Stem Uptake, 48 h) on Spinach Leaves and the Increased *N*-Feruloyl-3-methoxytyramine Content

treatment[a]	ppm	injury[b]	FMT, ppm[c]
acifluorfen control		+++	11 ± 3
AOA	4	+++	7 ± 3
	18	+	0.8 ± 0.7
	36	–	<0.5
$AgNO_3$	70	–	1.3 ± 0.5[d]
heat shock[e]		–	<0.5

[a] AOA introduced together with acifluorfen by stem uptake. $AgNO_3$ applied by leaf dip 24 h before acifluorfen. [b] Injury rated as none (–) to severe (+++) at 48 h in at least five experiments. AOA alone is phytotoxic at 180 but not at 72 ppm. $AgNO_3$ also counteracts acifluorfen injury at 40 and 140 ppm but is itself injurious at 280 ppm. AVG at up to 200 ppm is ineffective in counteracting acifluorfen injury. [c] Average and SD at 48 h in three experiments. FMT contents of controls were <0.5 ppm without herbicide treatment or with AOA alone (≤180 ppm) or $AgNO_3$ alone (≤280 ppm). [d] FMT content was 1-2 ppm with acifluorfen followed after 24 h by $AgNO_3$ at 140 or 280 ppm. [e] Heat shock at 50 °C for 25 s. Similar treatment at 45 °C is less effective, below 40 °C is ineffective, and at 55 °C or higher damages the tissue.

The first significant increases appear at <3 h for ethylene, 8–10 h for PAL, 10–15 h for FMT, and 15–20 h for ethane and malondialdehyde, at the time when necrosis becomes evident. The times vary somewhat from one experiment to another but the sequence is always the same and oxyfluorfen generally acts faster than acifluorfen. AVG pretreatment completely counteracts the ethylene production but none of the other effects of acifluorfen and oxyfluorfen.

Treatments Counteracting Acifluorfen and Oxyfluorfen Injury in Spinach and Soybean. In spinach, acifluorfen (5 ppm, stem uptake) and oxyfluorfen (10 ppm, leaf dip) lead to necrotic lesions after 24–48-h exposure to light. The phytotoxic effects of acifluorfen and oxyfluorfen are counteracted for at least 72 h by treatments with AOA (18–72 ppm, stem uptake), $AgNO_3$ (40–140 ppm, leaf dip), or heat shock (50 °C, 25 s) (Table IV) and by AOPP (100 ppm, stem uptake, preliminary experiment). Treatments effectively counteracting acifluorfen injury prevent the increased FMT content (Table IV).

AOA (40–60 ppm, stem uptake) also counteracts acifluorfen (10 ppm, stem uptake) injury to soybean seedlings.

Greenhouse experiments, which are not detailed here, revealed that addition of $AgNO_3$ to oxyfluorfen at 25:1 but not at 5:1 (w/w) markedly reduced DPE injury to both Bragg and Corsoy varieties of soybean with both preemergence and postemergence applications. This counteraction was not restricted to soybean since the postemergence herbicidal activity of oxyfluorfen to wild mustard [*Brassica kaber* (DC.) L. C. Wheeler] was also decreased by $AgNO_3$ in amounts equivalent to or up to 25 times that of oxyfluorfen.

DISCUSSION

The response of plants to DPE herbicides noted here resembles in several respects their "hypersensitive reaction" to microbial attack repoorted earlier (Kato and Misawa, 1976; Király, 1980). The same secondary products are often formed as phytoalexins and/or stress metabolites. Their formation is accompanied by increases in membrane permeability (Orr and Hess, 1982), lipid peroxidation with associated ethane and malondialdehyde formation (Sandmann and Böger, 1982a), ethylene liberation (Gorske and Hopen, 1978), and PAL activity (Kömives and Casida, 1982). Some or all of these disruptions are caused not only

by DPEs and microbial attack but also by a variety of DNA intercalating agents, inhibitors of protein synthesis, and alkylating agents (Cruickshank and Perrin, 1971; Hadwiger et al., 1974). Thus several different types of primary lesions lead to PAL induction and stress metabolite formation.

$AgNO_3$, heat shock, and the aminooxy compounds AOA and AOPP counteract DPE phytotoxicity, thereby helping to define the relation between ethylene liberation, PAL induction, phenylpropanoid biosynthesis, and phytotoxicity. Ethylene is an inducer of PAL (Hyodo et al., 1978) and toxic doses of the ethylene precursors ACC and ethephon increase the FMT content of spinach leaves. Acifluorfen- and oxyfluorfen-induced increases in FMT (when assayed) and phytotoxicity are counteracted by two anti-ethylene treatments, i.e., $AgNO_3$ which inhibits ethylene metabolism and action (Beyer, 1979) and heat shock which suppresses ethylene production (Field, 1981). They are also counteracted by two inhibitors of ethylene formation, other pyridoxal phosphate dependent processes, and PAL, i.e., AOA and AOPP (Amrhein and Wenker, 1979), but not by the potent and specific inhibitor of ethylene biosynthesis, AVG (Amrhein and Wenker, 1979). It therefore appears that DPE phytotoxicity is not related to ethylene production but instead to increased PAL activity (Kömives and Casida, 1982). Stress metabolites from PAL induction or other pathways, e.g., wyerone in broad bean and hemigossypol in cotton, are phytotoxic (Smith, 1982) and may therefore contribute to the DPE phytotoxicity. Alternatively, high PAL actiity may divert phenylalanine from protein synthesis or other critical cellular processes (Hoagland and Duke, 1981).

The ability to control synthesis of secondary plant metabolites with DPEs such as acifluorfen is of interest in several respects besides its possible contribution to their herbicidal action. It is useful in establishing biosynthetic pathways by increasing the rate of synthesis and content of secondary plant metabolites (Kömives and Casida, 1982). It provides a possible means to increase yields of some botanical pesticides, drugs, or pharmaceuticals with DPE treatment shortly before harvest. On the other hand, an increased content of secondary metabolites is not beneficial when they are present in crops and are toxic to mammals (Smith, 1982).

Acifluorfen is a selective herbicide for preemergence and postemergence control of broad-leaf weeds in soybeans and other large seeded legumes. The increase in content of stress metabolites from the use of acifluorfen appears to occur only at phytotoxic doses and may therefore pose no problem when DPE herbicides are used in a manner to avoid any crop injury.

ACKNOWLEDGMENT

Luis Ruzo of this laboratory and Sherri Ogden of the Department of Chemistry (University of California, Berkeley) performed the MS analyses. Helpful comments were provided by William Draper, Ian Holden, and Dierk Scheel of this laboratory. James Gerick of the Department of Plant Pathology (University of California, Berkeley) generously assisted in the antifungal assays.

Registry No. Acifluorfen·Na, 62476-59-9; glyceofuran, 78873-52-6; phaseollin, 13401-40-6; pisatin, 469-01-2; medicarpin, 32383-76-9; wyerone, 20079-30-5; xanthotoxin, 298-81-7; hemigossypol, 40817-07-0; oxyfluorfen, 42874-03-3; glyceollin I, 57103-57-8; glyceollin II, 67314-98-1; glyceollin III, 61080-23-7; silver nitrate, 7761-88-8; FMT, 83608-86-0; AOA, 645-88-5; AOPP, 42990-62-5.

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Received for review January 31, 1983. Accepted April 11, 1983. This study was supported in part by the National Institutes of Health (Grant PO1 ES00049).