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LIPOXYGENASE-DERIVED ALDEHYDES INHIBIT FUNGI PATHOGENIC ON SOYBEAN[1]

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Abstract—Several unsaturated aldehydes are produced from polyunsaturated fatty acids via the lipoxygenase pathway when soybean (*Glycine max*) plants are wounded mechanically or by pathogens. The effects of four of these aldehydes were examined on the growth of isolated fungal cultures of *Colletotrichum truncatum*, *Rhizoctonia solani*, and *Sclerotium rolfsii*. (*E*)-2-Hexenal, (*E*)-2-nonenal, and (*Z*)-3-nonenal inhibited the growth of *R. solani* and *S. rolfsii* at 35 μmol added per liter or greater when applied as volatiles, although higher levels were required for inhibition of *C. truncatum*. (*E*)-4-Hydroxy-2-nonenal was the most inhibitory compound when applied directly in the growth medium, but it had the least effect as a volatile.

Key Words—Aldehydes, (*E*)-2-hexenal, (*E*)-2-nonenal, (*Z*)-3-nonenal, (*E*)-4-hydroxy-2-nonenal, lipoxygenase, disease resistance, *Glycine max*, *Colletotrichum truncatum*, *Rhizoctonia solani*, *Sclerotium rolfsii*, soybean, fungal pathogens.

INTRODUCTION

Plant tissues respond to mechanical damage or attack by pathogens through physical and chemical changes, including rapid cell and tissue death, the formation of physical barriers, enzyme induction, and the production of secondary compounds (Misaghi, 1982). The enzyme lipoxygenase (LOX) catalyzes the oxygenation of the (*Z*,*Z*)-pentadiene moiety of polyunsaturated fatty acids

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(Gardner, 1991). LOX activity appears to be required for elicitation of hypersensitivity in plants exposed to fungal fatty acids such as arachidonic acid (Vaughn and Lulai, 1992). LOX expression has been shown to be stimulated in plant tissues undergoing infection or exposed to fungal chemical elicitors (Bostock et al., 1992; Ohta et al., 1991; Yamamoto and Tani, 1986). Lipid components of damaged cells may be oxidized via the LOX pathway to form lipid hydroperoxides, which rapidly break down to form many compounds, including aldehydes (Gardner, 1991). These aldehydes, although highly reactive, are sufficiently long-lived to diffuse from the sites of origin to reach extracellular targets (Esterbauer et al., 1991). Several of these aldehydes, including (*E*)-2-hexenal, (*Z*)-3-hexenal, and (*E*)-2-nonenal, were found to be inhibitory to seed germination (Bradow, 1991; Gardner et al., 1990), pollen germination (Hamilton-Kemp et al., 1991) and pathogenic fungi (Hamilton-Kemp et al., 1992) and bacteria (Deng et al., 1993). (*E*)-4-Hydroxy-2-nonenal (HNE) is also found in soybean preparations and may be formed from (*Z*)-3-nonenal (Gardner et al., 1991). A pathway to HNE from (*Z*)-3-nonenal has been recently described in *Vicia faba* microsomes (Gardner and Hamberg, 1993). HNE has been shown to be highly toxic to mammalian cells and tissues (Esterbauer et al., 1991). To the best of our knowledge, HNE has not been tested against plants or fungi.

Soybean (*Glycine max* L.) plants release many of these volatile C_6 and C_9 aldehydes derived from lipid oxidation when tissues are damaged (Gardner et al., 1991). These aldehydes may be extremely inhibitory to pathogenic fungi in enclosed airspaces such as those that occur in intracellular spaces in leaves or in air pockets under the soil surface. Thus aldehydes may play a major role in disease resistance. In this study we examine the activities of several aldehydes produced by soybean against the soybean pathogens, *Colletotrichum truncatum*, *Rhizoctonia solani*, and *Sclerotium rolfsii*. In particular, we wished to compare the toxicities of (*E*)-2-nonenal and (*Z*)-3-nonenal with that of the newly discovered LOX pathway metabolite, HNE.

METHODS AND MATERIALS

A fungal culture of *C. truncatum* (NRRL #13737; collected and identified by C.D. Boyette, USDA/ARS, Stoneville, Mississippi) was obtained from the culture collection at the National Center for Agricultural Utilization Research. Cultures of *R. solani* and *S. rolfsii* isolated from peanut were obtained from Dr. T. Brenneman, University of Georgia. All cultures were found to exhibit positive pathogenicity on soybean. All fungi were maintained in the dark at 25 $\pm$ 1°C on V-8 juice agar (pH 5.8), augmented with 0.3% $CaCO_3$.

(*E*)-2-Hexenal, (*E*)-2-nonenal, and DMSO used in the experiments were obtained from a supplier (Aldrich) and used without further purification. (*Z*)-3-

Nonenal was synthesized from (*Z*)-3-nonenol, as described previously (Gardner and Hamberg, 1993); and the structure was verified by ^{1}H NMR spectroscopy. HNE was synthesized from (*Z*)-3-nonenol, as previously described (Gardner et al., 1992), and its identity was verified by GLC of its *O*-benzyloxime-trimethylsilyloxy ether by comparison with a standard.

The bioassay system used to test the compounds as volatiles consisted of 75-ml jars into which sterilized V-8 juice medium (10 ml) was added. These jars were placed in 275-ml jars that contained one 5.5-cm filter paper disk (Whatman No. 1) at the bottom, to which appropriate amounts of the test compounds dissolved in DMSO were added (controls consisted of DMSO only). Aluminum foil cap liners were used to create an airtight seal. The total gas headspace in the system after the addition of all components was 250 ml. Bioassays were initiated by placing 7-mm plugs of media taken from the growing margins of 7-day-old cultures. These plugs were placed, mycelial surface down, on the surface of the agar in the 75-ml jars, and the DMSO stocks were then added to the 275-ml jars. Five jars of each treatment per fungus were incubated at 25 $\pm$ 1°C in the dark until control cultures reached the edges of the 75-ml jars (96 hr). Each experiment was repeated and results were pooled (N = 10) before statistical analysis of variance.

Compounds were also tested as suspensions in agar media to test the hypothesis that these compounds might be active when present in cell and tissue fluids as well as in the volatile phase. Appropriate amounts of the compounds were added to cooled sterilized V-8 medium in 9.0-cm plastic Petri dishes. Experiments were initiated with 7-mm plugs of mycelia that were taken from the growing margins of 1-week-old cultures and placed mycelial side down on the medium. The progress of fungal growth was determined by measuring the radial advancement of the mycelial mat (from the edge of the inoculum disk to one typical point on the growing margin) when the fastest-growing cultures reached the edge of the Petri plates. Treatments are reported as percentages of the (untreated) control. Each experiment consisted of five plates per treatment per fungus and was repeated. Results were pooled (N = 10) before statistical analysis of variance.

The volatilities of test compounds were assessed by GLC analyses of 1-ml headspace samples. Since volatility is usually decreased by its partition into the aqueous phase, headspace was analyzed under three conditions: (1) "neat" in a dry vessel (60 ml), (2) with the test compound applied directly to agar media (60 ml), and (3) with the test substance applied to filter paper in vessels containing agar media (250 ml). The test compounds were applied to the containers at the rate of 0.1 μl/ml headspace, and incubated at 26°C for 2–5 hr before sampling. Unexplained isomerization of (*E*)-2-alkenals in the neat treatments caused the headspace concentration of these compounds to approach a maximum at about 0.5 hr and to decrease with time. Thus, the (*E*)-2-alkenals were equil-

ibrated for only 0.5 hr. Headspace samples were taken with a 1-ml Pressure-Lok gas syringe (Precision Sampling Co., Baton Rouge, Louisiana), either through rubber septa (250 ml jars) or through Teflon mininert valves (Pierce Chemical, Rockford, Illinois) from the 60-ml vessels. Volatiles were separated and analyzed by a Spectra-Physics (San Jose, California) model SP-7100 gas chromatograph equipped with an open tubular capillary column (12 m × 0.22 mm) coated with SE-30 (Spectra-Physics). The carrier gas flow was about 1 ml/min, and the temperature was programmed from 100 to 125°C at 5°C/min for separation of the nonenal series [retention time (minutes) (*Z*)-3-nonenal, 1.3; (*E*)-2-nonenal, 1.7; HNE, 4.0]. For analysis of (*E*)-2-hexenal, the temperature was programmed from 45 to 55°C at 5°C/min (retention time, 1.6 min). The concentrations of volatiles in the test vessels were calculated from gas chromatographic analyses of known weights of aldehyde standards injected on the column in hexane solution.

RESULTS AND DISCUSSION

The bioassay system used in these experiments provided a convenient method for testing large numbers of volatile compounds rapidly against the growth of these fungal species. Rubber septa can be placed in the caps of the large jars, allowing headspace gases to be sampled for GLC analysis. Aluminum foil cap liners prevented loss of test compound and withstood autoclaving. Teflon cap liners did not satisfactorily withstand autoclaving.

The results obtained by exposing cultures of the three fungal species to the volatilized aldehydes are shown in Table 1. For *R. solani* and *S. rolfsii*, 35 μmol added per liter or greater of (*E*)-2-hexenal, (*E*)-2-nonenal, and (*Z*)-3-nonenal completely inhibited fungal radial growth. *C. truncatum* was more tolerant of these three compounds; 175 μmol added per liter were required for complete inhibition. As a volatile, HNE did not significantly inhibit either *R. solani* or *S. rolfsii* but the highest level (600 μmol added per liter) tested did decrease *C. truncatum* growth. This suggests that uptake or metabolism of these aldehydes by *C. truncatum*, a foliar pathogen, differs from these processes in the other two fungi, which primarily attack plant tissues below or at the soil level.

When applied directly to V-8 medium, the relative effects of the four aldehydes were somewhat different (Table 2). HNE, which had little effect as a volatile, became comparatively the most potent inhibitor of *C. truncatum* and *S. rolfsii* growth when it was included in the agar media. HNE in the media also caused significant growth inhibition of *R. solani* but was not as effective as the (*E*)-2-alkenals. *R. solani* was much more tolerant of (*E*)-2-nonenal, (*Z*)-3-nonenal, and HNE than were the other two fungi. *R. solani* was the only

TABLE 1. INHIBITION OF GROWTH OF FUNGI BY VOLATILE ALDEHYDES

Treatment (μmol added/liter headspace)	Fungal growth (% of control)[a]		
	C. truncatum	*R. solani*	*S. rolfsii*
Control	100.0 a	100.0 a	100.0 a
(*E*)-2-Hexenal			
3.5	85.0 a	100.0 a	60.8 b
35	35.8 d	0.0 b	0.0 d
175	0.0 e	0.0 b	0.0 d
875	0.0 e	0.0 b	0.0 d
(*E*)-2-Nonenal			
2.4	58.3 c	95.2 a	15.0 c
24	25.8 d	0.0 b	0.0 d
120	0.0 e	0.0 b	0.0 d
600	0.0 e	0.0 b	0.0 d
(*Z*)-3-Nonenal			
2.5	65.8 bc	98.7 a	45.0 b
25	64.2 bc	0.0 b	0.0 d
125	0.0 e	0.0 b	0.0 d
600	0.0 e	0.0 b	0.0 d
(*E*)-4-hydroxy-2-nonenal			
2.4	79.2 ab	100.0 a	100.0 a
24	76.7 abc	100.0 a	100.0 a
120	72.5 abc	100.0 a	100.0 a
600	69.9 bc	100.0 a	100.0 a

[a] Mean separation among all means by Duncan's multiple-range test, $P < 0.05$.

species significantly inhibited by the lowest level (0.4 μmol/ml medium) of (*E*)-2-hexenal. Both (*E*)-2-hexenal and (*E*)-2-nonenal completely suppressed all three species at levels of 2.2 and 1.5 μmol/ml, respectively. Again *S. rolfsii* was generally more sensitive and was the only fungal species to be inhibited by 0.3 μmol/mol HNE.

The higher level of tolerance of *C. truncatum* to the three highly volatile aldehydes [(*E*)-2-hexenal, (*E*)-2-nonenal, and (*Z*)-3-nonenal] may be due to a natural selection for strains of this fungi that are resistant to these compounds. Because these compounds are green leaf/cucumber volatiles produced by aerial parts of plants (such as leaves and the surfaces of fruit), *C. truncatum*, a phylloplane pathogen, would be expected to tolerate these common allelochemicals. Large increases in LOX activity were shown to be induced in soybean leaves by mechanical wounding or by spider mite damage (Hildebrand et al., 1989). Under normal conditions, the ability of a pathogen to colonize soybean leaves would appear to depend on its ability to tolerate lipoxygenase-derived compounds.

TABLE 2. INHIBITION OF FUNGI BY ALDEHYDES INCORPORATED IN MEDIA

Treatment (μ mol added/ml medium)	Fungal growth (% of controls)[a]		
	C. truncatum	*R. solani*	*S. rolfsii*
Control	100.0 a	100.0 a	100.0 a
(*E*)-2-Hexenal			
0.4	104.8 a	81.2 b	86.3 a
2.2	0.0 d	0.0 f	0.0 d
8.7	0.0 d	0.0 f	0.0 d
(*E*)-2-Nonenal			
0.3	32.2 c	89.1 ab	13.7 c
1.5	0.0 d	0.0 f	0.0 d
6.0	0.0 d	0.0 f	0.0 d
(*Z*)-3-Nonenal			
0.3	65.1 b	89.1 ab	64.1 b
1.6	0.0 d	61.8 c	0.0 d
6.3	0.0 d	23.6 de	0.0 d
(*E*)-4-Hydroxy-2-nonenal			
0.3	41.1 c	66.1 c	0.0 d
1.5	0.0 d	35.8 d	0.0 d
6.0	0.0 d	10.9 ef	0.0 d

[a]Mean separation among all values by Duncan's multiple-range test, $P < 0.05$.

The absence of detectable HNE in headspaces (Table 3) explains its lack of effect when applied as a volatile, despite previous reports of HNE toxicity to animal cells when applied in solution. In regard to the three test compounds with significant volatility, the extent of volatilization was (*E*)-2-hexenal >> (*Z*)-3-nonenal > (*E*)-2-nonenal (Table 3). However, the effect of the three compounds on growth suppression was roughly equivalent (Table 1), indicating a higher toxicity of the nonenals compared to (*E*)-2-hexenal. This was partially confirmed when these aldehydes were included in liquid media (Table 2), but inconsistent results were obtained in experiments with *R. solani*, where the nonenals were equally or less toxic than (*E*)-2-hexenal. In experiments employing liquid media, the headspace concentration of (*E*)-2-hexenal was reduced to 11% of the value found when the compound was analyzed neat. This is consistent with a previous observation (Gardner et al., 1991). Compared to (*E*)-2-hexenal, the headspace concentrations of the nonenals were reduced less by aqueous media (Table 3), reflecting the lower relative water solubility or partition coefficient of these aldehydes.

When the headspace volatiles were examined after 48 hr, it was discovered that the (*E*)-2-alkenals in the headspaces were little changed, but (*Z*)-3-nonenal had significantly decreased (data not shown). Since (*Z*)-3-nonenal readily autox-

TABLE 3. EFFECT OF BIOASSAY SYSTEM ON HEADSPACE CONCENTRATIONS OF ALDEHYDES

Aldehyde	Headspace concentration (μM)[a]		
	Condition A[b]	Condition B	Condition C
(*E*)-2-Hexenal	100.0	31.6	11.2
(*E*)-2-Nonenal	1.1	1.0	0.9
(*Z*)-3-Nonenal	4.6	1.5	1.6
(*E*)-4-Hydroxy-2-nonenal	0.0	n.d.[b]	n.d.

[a] All conditions: aldehydes were added to enclosed containers at the rate of 0.1 μl aldehyde/ml headspace and headspace volatiles were equilibrated at 26°C for 2–5 hr) [neat samples were equilibrated for only 0.5 hr to avoid the appearance of unexplained early-eluting isomer with the E-2-alkenals]. Condition A, aldehydes added neat to dry, clean vessel; condition B, method used in this study (aldehyde applied to filter paper in proximity to open agar container placed within a larger enclosed vessel); condition C, aldehyde applied directly to agar surface within closed container. Headspace gases were sampled via Teflon mininert valves in conditions A and C, via a rubber septa in condition B.

[b] n.d., not determined.

idizes to (*E*)-4-hydroperoxy-2-nonenal (Gardner and Hamberg, 1993), the material remaining in the container was extracted with $CHCl_3$–CH_3OH (2:1, v/v), and the recovered material was reduced with triphenylphosphine (TPP). Subsequent conversion to the *O*-benzyloxime-trimethylsilyloxy derivative followed by GLC showed the presence of HNE from reduction of the hydroperoxide by TPP. This conversion may partially explain the observed effect of (*Z*)-3-nonenal on the growth of fungi.

It has been previously shown that many volatile compounds, including LOX-derived aldehydes, stimulated spore germination in many fungal species (French, 1992). Fungistasis, the inhibition of germination of fungal propagules by soil, has been attributed, in part, to volatile compounds present in the soil (Hora and Baker, 1970; Liebman and Epstein, 1992). In our study none of the levels of any of the compounds tested stimulated fungal growth, but it is conceivable that lower concentrations of these aldehydes would be stimulatory. In fact, several aldehydes not examined in this study (hexanal, nonanal) promoted hyphal growth of *Alternaria alternata* and *Botrytis cinerea* (Hamilton-Kemp et al., 1992). It is interesting to note that both (*E*)-2-hexenal and (*E*)-2-nonenal only inhibited growth in the latter study. This may indicate that certain plant-derived volatiles act by stimulating the germination of the relatively resistant spores, allowing other volatiles to act against the more vulnerable hyphae.

In conclusion, several unsaturated aldehydes derived from LOX-catalyzed lipid oxidation were found to inhibit the growth of isolated fungal cultures of *C. truncatum*, *R. solani*, and *S. rolfsii*. These aldehydes were inhibitory both

as volatiles and as media constituents. In general, *R. solani* cultures were more resistant to volatilized aldehydes than were the other two fungi. *C. truncatum* was the most tolerant to the aldehydes added to the media. HNE, which was not inhibitory as a volatile, was the most inhibitory aldehyde tested when added to the media.

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