Structure–Antifungal Activity Relationships among Volatile C_6 and C_9 Aliphatic Aldehydes, Ketones, and Alcohols

Roger A. Andersen,*,†,‡ Thomas R. Hamilton-Kemp,§ David F. Hildebrand,‡ Charles T. McCracken, Jr.,‡,§ Randall W. Collins,§ and Pierce D. Fleming†,‡

Agricultural Research Service, U.S. Department of Agriculture, and Departments of Agronomy and Horticulture, University of Kentucky, Lexington, Kentucky 40546-0091

Antifungal potentials of volatile oxygenated alkyl compounds were determined by bioassay of *Alternaria alternata* germ tube growth. Compounds were naturally occurring lipoxygenase enzyme system products or related to them chemically. Results were based on headspace concentrations of test compounds. Among six- and nine-carbon aldehydes and ketones related to hexanal, nonanal, 3-hexanone, and 2-nonanone, respectively, the presence of an α,β-unsaturated bond adjacent to the carbonyl moiety generally enhanced antifungal activity. Among six-carbon alcohols related to 1-hexanol, however, an α,β-unsaturated bond enhanced antifungal activity only slightly. Abundant, naturally occurring (*E*)-2-hexenal that higher plants emit as a response to wounding tested negatively for mutagenicity; a volatile compound modification of the Ames test with several strains of *Salmonella typhimurium* was used.

Keywords: *Antifungal, Alternaria, lipoxygenase products, bioassay, volatiles, aldehydes, ketones, α,β-unsaturated, (E)-hexenal, mutagenicity*

INTRODUCTION

(*E*)-2-Hexenal was first identified as an active volatile antifungal compound in *Gingko biloba*, a tree known for its resistance to pests and disease (Major et al., 1960). Maruzzella et al. (1961) demonstrated in vitro antifungal properties of vapors and suggested that the groups of chemicals tested had the following order of decreasing antifungal activity: acids, aldehydes, alcohols, ethers, etc. The compounds tested generally had more than six carbon atoms. Nandi (1977) demonstrated for fungal pathogens of wheat that inhibitions of linear growth of mycelia and dry matter production occurred after exposures to several classes of volatile compounds listed in decreasing order of activity: aldehydes, esters, terpenoids, and ketones. Urbasch (1984) determined that (*E*)-2-hexenal had lower minimal fungal-growth-inhibiting concentrations than hexanal, 1-hexanol, (*E*)-2-hexen-1-ol, and (*Z*)-3-hexen-1-ol as determined for several species. Gueldner et al. (1985) demonstrated that the volatiles (*E*)-2-hexenal, 2,4-hexadienal, furfural, β-ionone, and 1-nonanol and some synthetic analogues of these compounds inhibited the growth of *Aspergillus flavus* (CP-22) as measured by mycelial growth. The order of activity of these compounds was suggested to be aldehydes > ketones > alcohols. Zeringue and McCormick (1989) compared several C_6–C_9 aldehydes, ketones, and alcohols and determined that alkenals, especially (*E*)-2-hexenal, were the most effective inhibitors of *A. flavus*.

Although known quantities of liquid chemicals were used to supply the vapors in the exposure chambers in the above studies comparing the relative antifungal activities of compounds in the gas phase, headspace concentrations of the test compounds were not measured directly for the quantitative comparisons. Thus, different volatilities among compounds were not accounted for in their bioassays. In the initial study of structure–activity relationships between directly measured gaseous compounds and fungal growth inhibition carried out in our laboratories, it was shown that equivalent headspace concentrations of the α,β-unsaturated aldehyde, (*E*)-2-hexenal, were considerably more active in their inhibition of hyphal growth of two fungal pathogens, *Alternaria alternata* and *Botrytis cinerea*, than the corresponding saturated aldehyde, hexanal (Hamilton-Kemp et al., 1992). Some of the most active antifungal compounds determined in previous studies [e.g. (*E*)-2-hexenal, etc.] are known to form in relatively large amounts in plant tissues in response to wounding and the subsequent action of the lipoxygenase enzyme system (LOX) involving lipid oxidation. Hildebrand (1989) suggested that LOX-mediated products including aliphatic aldehydes, ketones, and alcohols are involved in plant defense.

Aliphatic aldehydes and, to a somewhat lesser extent, ketones are relatively reactive compounds containing a polarized carbon–oxygen double bond. The chemical nature of α,β-unsaturated aldehydes and some of their toxicological effects based on their ability to function as direct-acting alkylating agents were reviewed by Schauenstein et al. (1977) and Witz (1989). The carbonyl carbon is an electrophilic site and reacts easily with nucleophiles. Under physiological conditions nucleophilic attack on the carbonyl moiety by primary amines, on the one hand, and thiols and possibly alcohols, on the other, results in the formation of substituted imines called Schiff bases and hemiacetals, respectively. Attack of a second amine or thiol on the initial adducts can result in protein–protein, DNA–protein, or DNA–DNA cross-linking (Feron et al., 1991). When α- and β-carbons adjacent to carbonyl moieties possess a double bond, the molecule becomes even more reactive. Because of its conjugation with the carbonyl group, the β-carbon becomes positively polarized and will be the site of nucleophilic attack.

Among volatile naturally occurring α,β-unsaturated carbonyl compounds, (*E*)-2-hexenal is the predominant volatile component which has been found in vegetative portions of virtually all plant species studied. Since this

* Author to whom correspondence should be addressed.
† Agricultural Research Service.
‡ Department of Agronomy.
§ Department of Horticulture.

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compound exhibits inhibitory or toxic effects against pathogens, it may find applications in pest control through fumigation or other application to reduce pathogens or pests on plants or food products. On the basis of these considerations, we evaluated the safety of (*E*)-2-hexenal using the Ames test. Further testing of the compound for potential practical benefits in reducing pathogen populations and inhibiting growth may depend on establishing that it either is nonmutagenic or has low mutagenic potential as has been reported for many natural products which commonly occur in the diet or environment (Feron et al., 1991; Eder et al., 1992).

The purpose of this investigation is to quantitatively determine and compare effects on the germination of *A. alternata* spores (via measurement of germ tube elongation) of carbon chain length, α,β-unsaturation, and other positional unsaturation sites of directly determined headspace concentrations of aliphatic aldehydes, ketones, and alcohols. The naturally occurring compounds investigated are known LOX-mediated products or chemicals structurally related to these kinds of compounds. In addition, we were interested in determining whether α,β-unsaturated compounds such as the prototype (*E*)-2-hexenal were mutagenic in the vapor phase, a property that could preclude the use of formulations of these compounds as pesticides.

MATERIALS AND METHODS

Fungal Culture and Bioassay. Isolates of *A. alternata* (Fr.) Keissl. from lesions of tobacco leaves were transferred, cultured, and maintained on V-8 juice agar saturated with $CaCO_3$ at pH 6.2 (Spurr, 1973). Cultures were kept at approximately 23 °C under continuous fluorescent lighting to stimulate sporulation. The system used to determine effects of volatiles on conidiospores from *A. alternata* was described by Hamilton-Kemp et al. (1992). All glassware was sterilized prior to bioassay, and the spores were handled aseptically. A 9-cm Petri dish containing a half-hole rubber septum fitted through a hole in the center of the cover was used to house separately a 5-cm coverless Petri dish containing a 1-cm^3 2% water agar block and a 1-cm-diameter glass sample dish that contained 1 mL of water.

For the bioassay, spores were spread on the surface of the agar block and 10 μL of a solution of the volatile test compound was placed in the sample dish containing water. The cover of the 9-cm Petri dish was then placed over the system (internal volume, 120 mL), which was immediately wrapped with Parafilm. After 4–6 h, germ tube lengths were measured using a microscope with a net micrometer ocular at 100× magnification. [A drop of neat (*E*)-2-hexenal could be added to the 9-cm Petri dish to stop germ tube growth and permit measurements when convenient.] Standard errors of the mean were calculated. ANOVA was also performed on results, and Fisher's least significant difference (lsd) test was used only where the *F* test was significant at the $P = 0.05$ level of probability (Einot and Gabriel, 1975).

Chemicals. Synthetic samples of volatile compounds investigated in the bioassays included the following: (aldehydes) hexanal, (*E*)-2-hexenal, nonanal, (*E*)-2-nonenal, (*Z*)-6-nonenal, (*E,E*)-2,4-nonadienal, (*E,Z*)-2,6-nonadienal; (ketones) 3-hexanone, (*E*)-4-hexen-3-one, 2-nonanone, (*E*)-3-nonen-2-one; (alcohols) 1-hexanol, (*E*)-2-hexen-1-ol, (*Z*)-3-hexen-1-ol. The listed chemicals were purchased from Aldrich (Milwaukee, WI) except for (*E,E*)-2,4-nonadienal, (*E,Z*)-2,6-nonadienal, and (*Z*)-6-nonenal, which were obtained from Bedoukian Research, Inc. (Danbury, CT). The chemicals were of the best commercial grades available and were used without further purification. Stock solutions of the chemicals for use in the bioassay were made up at 10% (w/w) in 1,2-propanediol (Sigma, St. Louis, MO) except for nonanal, which was made up in paraffin oil 335/350 obtained from Fisher (Pittsburgh, PA). Stock solutions were used directly or after serial dilutions to obtain 1% and 0.1% solutions (w/w). Due to low volatility, the highest concentration of alcohols tested were neat and the middle and lowest concentrations were 10% and 1%, respectively, made up as solutions in 1,2-propanediol (w/w) as above.

Quantitative Estimation of Compounds in Headspace. For quantitative estimation of compounds in the vapor phase (headspace), 9-cm Petri dishes containing the microcup with water and the solution of test compound described above (but without the 5-cm dish and agar block) were used. The system was wrapped with Parafilm and incubated at 24 ± 1 °C for a specified period. Subsequently, a sample of headspace vapor was withdrawn (and transferred) from the system through the septum with a syringe and injected into a GC injection port using either of the following two protocols for syringe equilibration and transfer: (1) for medium and high concentrationns of headspace volatiles, a 300-μL sample was withdrawn with a 500-μL gastight syringe and discharged back into the 9-cm Petri dish, and this step was repeated once. A third 300-μL sample was withdrawn and 50 μL of it was discharged to waste outside the Petri dish before the remaining 250 μL was injected into the GC. (2) For low concentrations of headspace volatiles (i.e., some C_9 compounds), a 1.0-mL sample was withdrawn with a 10-mL gastight syringe and discharged within the 9-cm Petri dish, and this step was repeated once. A third 1.0-mL sample was withdrawn and injected into the GC. A Varian 3700 GC equipped with a 30 m × 0.53 mm × 1-μm film DB-Wax (polyethylene glycol) column and a 1 m × 0.53 mm fused silica precolumn was used for direct injection with the following operating conditions: inlet, 220 °C; column oven, 50 °C for 5 min, then programmed at 3 °C/min to 150 °C; detector, FID at 240 °C; helium flow rate, 6 mL/min. Concentrations of compounds at three different levels generated gas-phase concentrations that were sampled from the headspace of Petri dish bioassays during incubations and estimated by GC analysis. In the quantitations, GC area unit responses of test compounds were compared to response factors expressed as GC area units per unit weight of compound determined for the same carbon chain length reference compound, i.e., either (*E*)-2-hexenal or (*E*)-2-nonenal. Response factors for the reference compounds were determined using a 2-L static dilution bottle following an EPA method (Winberry et al., 1988) employing argon for dilution. Headspace results for a given level of volatile compound were determined as a weighted mean value ± standard deviation of separate GC determinations at eight time periods (designated t_1–t_8) representing incubations at 0.25, 0.50, 1, 2, 3, 4, 5, and 6 h performed on two replicates. Thus, a weighted mean headspace (exposure) concentration was determined using the following calculation for the 6-h incubations: $0.25c_1t_1 + 0.25c_2t_2 + 0.5c_3t_3 + 1.0c_4t_4$, ..., $1.0c_8t_8$, where c is the headspace concentration in nanomoles per liter of air and t is the time elapsed (hours) from start of incubation.

For Ames tests (described below), the vapor-phase concentration of (*E*)-2-hexenal in a 9-L desiccator maintained at 37 °C was measured at intervals over a 6-h period using methods similar to those for the Petri plates. The weighted mean values of vapor-phase concentrations in two replicate setups were determined as for the Petri dishes. The value for 5 μL of (*E*)-2-hexenal was determined by interpolation based on the relationship of vapor concentrations from 1 and 10 μL of compound; the value for 0.1 μL was calculated by assuming complete vaporization in the desiccator.

Mutagenicity Studies. Assays with *Salmonella typhimurium* TA 97a, TA 98, TA 100, TA 102, and TA 104 (kindly provided by Carol Wehr in the laboratory of Dr. Bruce Ames, University of California, Berkeley, CA) were carried out for (*E*)-2-hexenal by the desiccator assay modification for volatile compounds (Simmon et al., 1977; Simmon, 1981) based on the Ames test method (Maron and Ames, 1983). Tester strains were cultured in Oxoid broth no. 2 at 37 °C in a gyrating incubator. Test plates containing agar were overlaid with 2 mL of top agar containing 0.1 mL of bacterial suspension and 0.5 mL of phosphate buffer (pH 7.4). The plates without lids were placed on a shelf in a 9-L desiccator containing a magnetic stir bar. The desiccator was set on a magnetic stir plate inside an incubator at 37 °C, and the bacteria were exposed to vapors for 8 h with continuous stirring.

Subsequently, the lids were placed on the plates which were then wrapped with Parafilm and held at 37 °C for 40 h. Colonies were then counted with a Darkfield Quebec Model 3330 colony counter. It was impractical to measure 1 and 0.1 μL of (*E*)-2-

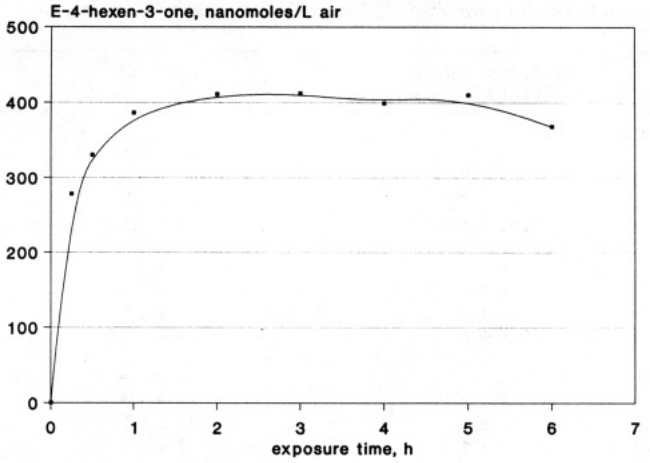

Figure 1. Headspace concentrations of (*E*)-4-hexen-3-one in 9-cm Petri plate during 6-h exposure period.

hexenal; thus, solutions of the compound in CH_3CN were prepared, and 10 μL of these solutions was bioassayed. In tests with an S-9 activation system, freeze-dried samples (Molecular Toxicology, Inc., Annapolis, MD) were rehydrated and 100 μL of S-9 mix containing 20 μL of S-9 was added to the top agar.

Using 800 μL of CH_2Cl_2 (without S-9) as a positive control in the desiccator test, we obtained 823 revertants of TA 100 per plate (controls had 172 revertants), whereas Simmon et al. (1977) obtained ca. 1200 revertants per plate. Positive controls using nonvolatile 2-aminofluorene added to the top agar and with S-9 present were active; revertants per plate were as follows: TA 97a [treated (tr) = 1037, untreated (untr) = 125]; TA 98 (tr = 2627, untr = 37); TA 100 (tr = 1036, untr = 145); TA 102 (tr = 750, untr = 508); and TA 104 (tr = 909, untr = 279).

RESULTS AND DISCUSSION

Typical Plot Illustrating Headspace Concentrations of Exposures to an Oxygenated Alkyl Compound during Bioassay. (*E*)-4-Hexen-3-one concentrations (from the intermediate of the three liquid levels used) as a function of fungal exposure times are illustrated as a best curve fit plot in Figure 1. Data points on the graph were average values obtained from two replicate samples. The largest concentration changes for the C_6 and C_9 compounds used in the bioassays occurred from 0 to 1 h. The plot of the illustrative compound and the other aldehydes, ketones, and alcohols used in the bioassays tended to plateau and remain stable from about 1 to 6 h as illustrated in the figure. However, 2-nonanone reached a peak level at 2–3 h and then declined during the sampling period. Vapor-phase concentrations of volatile compounds for a completed bioassay (presented in Table 1) were determined as weighted mean values ± standard deviation of 16 separate determinations at incubation times throughout the bioassay as described under Materials and Methods.

Effects of Oxygenated Alkyl Headspace Compounds on Fungal Germ Tube Elongations. Seven aldehydes were used to compare the contributions of structural differences of C_6 and C_9 compounds in headspace on *A. alternata* germ tube elongations after 6-h exposures in the bioassay protocol. Compounds investigated were selected on the basis of carbon chain length and presence or absence of α,β or other positional double bonds. Results for aldehydes in Table 1 were compiled for controls without aldehydes in addition to three headspace concentrations for each aldehyde determined by the quantitative gas chromatographic–static dilution bottle estimation method described under Materials and Methods. Each aldehyde suppressed germ tube elongation of *A. alternata* during the 6-h bioassay upon exposure to at least two of the three headspace concentrations used. However, the lowest level of each aldehyde's concentration was ineffective in suppressing germ tube length in each case. It should be noted that headspace concentrations resulting from the same volume of liquid aldehyde were different for the individual aldehydes and direct comparisons of headspace concentrations and germ tube length could not be made among individual aldehydes from Table 1 values.

Four ketones were used to determine the contributions of structural differences of C_6 and C_9 compounds in headspace on *A. alternata* germ tube lengths after 6-h exposures of the fungal spores in the bioassay procedure. The C_6 and C_9 saturated ketones used were compared with an α,β-unsaturated counterpart in each case. Results are given in Table 1 and were compiled for one control without ketone in addition to three headspace concentrations for each ketone determined as in the case of the aldehydes. Three of the four ketones suppressed germ tube elongations at a minimum of at least one of the three vapor-phase concentrations. However, the C_6 ketone, 3-hexanone, was ineffective in suppressing germ tube elongation at each level of headspace concentration used. Headspace concentrations resulting from the same volume of liquid ketone were different for the individual compounds. Thus, direct comparisons of headspace concentration and germ tube length could not be made among individual ketones, which was the case for the volatile aldehydes.

Three alcohols were used to determine the contributions of structural differences of C_6 compounds in headspace on *A. alternata* germ tube elongations in the bioassay procedure. Alkyl alcohols of C_9 structure were not used because of their relatively nonvolatile characteristics. Compounds were selected on the basis of the presence or absence of an α,β carbon–carbon double bond and geometrical isomerism. Results are given in Table 1 for sets consisting of a control and three headspace concentrations for each alcohol investigated. Each alcohol suppressed germ tube elongation when used at vapor-phase concentrations that were either maximal or at the intermediate level of the three levels used. Headspace concentrations resulting from the same volume of liquid alcohol were more similar among the individual alcohols than was the case for the aldehydes and ketones.

Quantitative Estimations of Antifungal Activities of Oxygenated Alkyl Headspace Compounds. The relative antifungal activities of individual compounds were calculated and derived in terms of headspace concentrations that caused a 50% reduction in the germ tube elongation of *A. alternata* elicited by the respective bioassay control. These values were designated by the acronym GTL_{50} and were determined from the sets of results for headspace concentrations of individual oxygenated alkyl compounds and germ tube lengths of *A. alternata* as given in Table 1. An *x*–*y* plot of germ tube lengths vs headspace concentrations for a given compound was used to generate a best curve fit by simple regression. A GTL_{50} value was obtained from the curve by interpolation.

The GTL_{50} values of C_6 and C_9 aldehydes, ketones, and alcohols are given in Table 2. Aldehyde, ketone, and alcohol values ranged from 1 to 54, from 7 to >23, and from 50 to 72 nmol × 10^{-2}/L of air, respectively. These compounds were also ranked in the order of decreasing antifungal activity as determined by their relative GTL_{50} values compared within their respective group. The most biologically active aldehydes or ketones contained an α,β-unsaturated double bond. The presence or absence of

Table 1. Effects of Vapor Concentrations of Aldehydes, Ketones, and Alcohols on Fungal Germ Tube Lengths Relative to Controls[a,b]

compd	α,β-unsaturated site in molecule	headspace concn,[c] nmol × 10^{-1}/L of air	germ tube length, μm × 10^{-1}
		Aldehydes	
n-hexanal	no	0	12 ± 6 A
		12 ± 2	12 ± 7 A
		117 ± 29	8 ± 3 B
		1221 ± 104	<1 ± <1 C
(*E*)-2-hexenal	yes	0	16 ± 8 A
		6 ± 1	15 ± 7 A
		45 ± 8	4 ± 2 B
		580 ± 80	1 ± <1 C
n-nonanal	no	0	16 ± 8 A
		1 ± <1	16 ± 7 A
		12 ± 3	11 ± 5 B
		117 ± 19	1 ± <1 C
(*E*)-2-nonenal	yes	0	19 ± 9 A
		4 ± <1	18 ± 8 A
		27 ± 4	0 B
		212 ± 88	0 B
(*Z*)-6-nonenal	no	0	11 ± 4 A
		2 ± <1	11 ± 4 A
		22 ± 8	6 ± 3 B
		89 ± 39	<1 ± 0 C
(*E*,*E*)-2,4-nonadienal	yes	0	12 ± 4 A
		2 ± <1	12 ± 4 A
		25 ± 3	3 ± 3 B
		143 ± 21	0 C
(*E*,*Z*)-2,6-nonadienal	yes	0	14 ± 5 A
		1 ± <1	12 ± 4 A
		22 ± 4	3 ± 2 B
		214 ± 34	0 C
		Ketones	
3-hexanone	no	0	9 ± 3 NS
		10 ± 1	9 ± 3 NS
		107 ± 14	9 ± 3 NS
		1182 ± 136	9 ± 3 NS
(*E*)-4-hexen-3-one	yes	0	15 ± 4 A
		4 ± <1	12 ± 4 B
		39 ± 3	6 ± 3 C
		402 ± 21	0 D
2-nonanone	no	0	17 ± 3 A
		2 ± 1	16 ± 3 A
		44 ± 14	16 ± 3 A
		342 ± 146	4 ± 2 B
(*E*)-3-nonen-2-one	yes	0	16 ± 5 A
		2 ± <1	17 ± 4 A
		22 ± 3	11 ± 5 B
		192 ± 36	0 C
		Alcohols	
1-hexanol	no	0	11 ± 5 A
		22 ± 6	11 ± 6 A
		246 ± 27	8 ± 4 B
		1594 ± 144	0 C
(*E*)-2-hexen-1-ol	yes	0	16 ± 7 A
		14 ± 2	14 ± 6 A
		155 ± 20	10 ± 5 B
		1424 ± 140	0 C
(*Z*)-3-hexen-1-ol	no	0	14 ± 5 A
		12 ± 1	14 ± 6 AB
		132 ± 14	12 ± 4 B
		1106 ± 58	0 C

[a] Mean values ± SD are given for headspace concentrations and fungal germ tube lengths. [b] Mean values for germ tube lengths in a vertical column subset (by compd) followed by no corresponding letter are significantly different at $P = 0.05$. [c] Concentrations of compounds at three different levels generated gas-phase concentrations that were sampled from the headspace of Petri dish bioassays during incubations and estimated by GC analysis (see Materials and Methods).

this kind of unsaturated bond did not affect the alcohols to as great an extent, and it appeared that a carbonyl group adjacent to the double bond was required for the larger increases of antifungal activity observed compared

Table 2. Relative Antifungal Activities of Volatile C_6 and C_9 Oxygenated Hydrocarbons vs *A. alternata*

compd (rank in order of decreasing antifungal activity)	headspace concn causing 50% reduction of control germ tubelength (GTL_{50}),[a] nmol × 10^{-2}/L of air
Aldehydes	
hexanal (7)	54
(*E*)-2-hexenal (6)	10
nonanal (5)	6
(*E*)-2-nonenal (1)	1
(*Z*)-6-nonenal (4)	4
(*E*,*E*)-2,4-nonadienal (3)	3
(*E*,*Z*)-2,6-nonadienal (2)	2
Ketones	
3-hexanone (4)	*b*
(*E*)-4-hexen-3-one (1)	7
2-nonanone (3)	23
(*E*)-3-nonen-2-one (2)	8
Alcohols	
1-hexanol (3)	72
(*E*)-2-hexen-1-ol (1)	50
(*Z*)-3-hexen-1-ol (2)	55

[a] An *x*–*y* plot of germ tube lengths vs headspace concentrations for a given compound was used to generate a best curve fit. A GTL_{50} value was obtained from the curve by interpolation. [b] 3-Hexanone concentrations ranged up to 1.2 μmol/L of air but did not affect germ tube lengths.

to the saturated aldehydes and ketones, namely, hexanal, nonanal, 3-hexanone, and 2-nonanone. Among the aldehydes investigated, (*E*)-2-nonenal had the highest degree of antifungal activity with decreasing effectiveness exhibited by (*E*,*Z*)-2,6-nonadienal, (*E*,*E*)-2,4-nonadienal, (*Z*)-6-nonenal, nonanal, (*E*)-2-hexenal, and hexanal. It is probable that the presence of an α,β-unsaturated double bond increases the electrophilic properties of the carbonyl compounds that contain this bond structure compared to their saturated counterparts (Schauenstein et al., 1977). Consequently, the inhibitory properties of these compounds and their effectiveness against *A. alternata* may be increased due to their increased propensity to react with thiols and amino groups of the target fungi. (*Z*)-6-Nonenal possesses a double bond that is not adjacent to the carbonyl functional group. Therefore, it does not meet the requirement for increased electrophilic properties of the carbonyl group that is met by the more strongly inhibitory (antifungal) structural analogues such as (*E*)-2-nonenal.

Our results indicate that, at the quantities investigated, C_9 aldehydes and ketones are generally more potent than their C_6 counterparts as antifungal agents vs *A. alternata* spore germination and subsequent germ tube elongation. These findings are in agreement with our earlier results concerning responses of *A. alternata* to some of these agents (Hamilton-Kemp et al., 1992). It is noteworthy, however, that C_6 aldehydes were more effective than their C_9 counterparts vs *B. cinerea* (Hamilton-Kemp et al., 1992).

Interestingly, Harman et al. (1980) found that volatiles produced by seeds under conditions that accelerated seed deterioration stimulated germination of spores of *A. alternata* and other fungi. In a recent review, French (1992) noted that certain compounds can function as binary messages causing fungal growth stimulation or inhibition depending on the exposure levels. In fact, we found that nonanal and other saturated carbonyls (but not α,β-unsaturated carbonyls) stimulated aerial growth of hyphae from cultures of *A. alternata* when tested at low concentrations for several days (unpublished results).

Aldehydes as headspace components were particularly strong inhibitory agents for *A. alternata* compared to analogous alkyl ketones and alcohols investigated. Results of several earlier studies indicated that oxygenated alkyl compounds as volatiles at unspecified headspace concentrations were effective as antifungal agents (Maruzzella et al., 1961; Nandi, 1977; Lyr and Banasiak, 1983; Urbasch, 1984; Gueldner et al., 1985; Zeringue and McCormick, 1989) and that the order of decreasing activity of the volatiles was generally aldehydes, ketones, and alcohols. In our previous study (Hamilton-Kemp et al., 1992) it was demonstrated that headspace concentrations of the α,β-unsaturated aldehyde, (*E*)-2-hexenal, were considerably more active in their inhibition of hyphal growth (germ tube elongation) of two fungal pathogens, *A. alternata* and *B. cinerea*, than the corresponding saturated aldehyde, hexanal.

Iriye et al. (1988) showed that α,β-unsaturated bisenals and biseones were stronger growth inhibitory compounds than monoenals or monoenones when tested against the yeast *Rhodotorula gracilis*. Unlike the compounds investigated in our present study, the bis compounds are not thought to be constituents of plant tissues.

(*E*)-2-Hexenal Mutagenicity Studies (Ames Test). The desiccator test system for volatile compounds provided a method for evaluating the mutagenic properties of (*E*)-2-hexenal, an important prototype of α,β-unsaturated carbonyl compounds. (*E*)-2-Hexenal is ubiquitous in vapors of damaged plants and is toxic to microorganisms. Mutagenic properties of the vapors might preclude future use of this or related compounds in pest control.

Tests with 26 μmol/L of air (100 μL of liquid) of (*E*)-2-hexenal in the desiccator showed that growths of *S. typhimurium* strains TA 97a, TA 98, TA 100, and TA 102

Table 3. Bioassay for Mutagenic Response of *S. typhimurium* after Exposure to (*E*)-2-Hexenal (Leaf Aldehyde) in the Vapor Phase–Ames Desiccator Test

bacterial strain	S-9	no. of His^+ revertants per plate ± SD: treatment (control) 0.06 μmol of (*E*)-2-hexenal/L of air	0.6[a] μmol of (*E*)-2-hexenal/L of air	3 μmol of (*E*)-2-hexenal/L of air	6 μmol of (*E*)-2-hexenal/L of air	26 μmol of (*E*)-2-hexenal/L of air
TA 97a	–		118 ± 14 (167 ± 5)		42 ± 13 (130 ± 10)	0 (98 ± 11)
TA 97a	+		99 ± 2 (109 ± 7)	81 ± 12 (126 ± 5)	56 ± 8 (98 ± 10)	
TA 98	–		24 ± 1 (22 ± 4)		23 ± 4 (22 ± 3)	0 (31 ± 3)
TA 100	–		203 ± 10 (188 ± 3)		18 ± 8 (124 ± 3)	0 (146 ± 4)
			172 ± 21 (195 ± 6)[b]			
TA 100	+		155 ± 12 (174 ± 18)	118 ± 17 (126 ± 13)	167 ± 15 (108 ± 1)	
TA 102	–		425 ± 47 (416 ± 20)		481 ± 22 (451 ± 42)	0 (378 ± 3)
TA 104	–	188 ± 4 (204 ± 3)	131 ± 8 (212 ± 6)	122 ± 1 (177 ± 14)	135 ± 2 (159 ± 6)	
TA 104	+	221 ± 7 (273 ± 5)	157 ± 17 (227 ± 9)	121 ± 9 (223 ± 11)	116 ± 10 (227 ± 5)	

[a] Estimated average vapor-phase quantity of (*E*)-2-hexenal in 9-L desiccator (see Materials and Methods); the quantities of liquid (*E*)-2-hexenal placed in the desiccator to obtain 0.6, 6, and 26 μmol/L of air were 1, 10, and 100 μL, respectively. The 3 μmol/L value corresponding to 5 μL of test liquid was obtained by interpolating the results from 1 and 10 μL; 0.06 μmol/L was obtained by assuming complete evaporation of 0.1 μL of (*E*)-2-hexenal. [b] Test done with 3-fold more bacterial than standard Ames test (Eder et al., 1992).

were completely inhibited (Table 3). Tests were subsequently conducted using 6 μmol/L (10 μL of liquid) and 0.6 μmol/L (1 μL of liquid) of the compound, but mutagenicity was not demonstrated. TA 100 used at 3-fold higher bacterial levels than normal (Eder et al., 1992) did not cause increased mutagenicity using 0.6 μmol of (*E*)-2-hexenal/L. This technique was recently employed to overcome compound toxicity in solution. In addition, a fifth tester strain, TA 104, sensitive to carbonyl compounds, was tested at 6 μmol/L and lower concentrations. None of the strains with the exception of TA 104 were significantly inhibited by (*E*)-2-hexenal at 0.6 μmol/L; TA 104 was also tested with 0.06 μmol/L (0.1 μL of liquid), but no mutagenicity was observed.

Activation of (*E*)-2-hexenal by S-9 mix did not increase colony counts, except for TA 100 at 6 μmol/L, in which counts increased with S-9 (Table 3). In the latter test, S-9 may have reacted with (*E*)-2-hexenal to diminish its inhibitory properties.

On the basis of the results with the five tester strains, with and without S-9, (*E*)-2-hexenal was not mutagenic to *S. typhimurium* when tested in the vapor phase. However, Marnett et al. (1985) and Eder et al. (1992) found that, by using a modification of the Ames test, (*E*)-2-hexenal was mutagenic in the liquid phase. When we repeated the liquid-phase test of Marnett et al. which included a glutathione chase, we also found that (*E*)-2-hexenal was mutagenic. (*E*)-2-Hexenal was relatively toxic to *S. typhimurium* in both the present studies and those of Marnett et al. (1985) and Eder et al. (1992). It is not clear at this point why there were differences in mutagenesis of this compound between the vapor- and liquid-phase assays.

Conclusions. Among six- and nine-carbon volatile oxygenated alkyl compounds, the aldehydes and ketones most inhibitory to *A. alternata* spore germination and subsequent germ tube elongations contained an α,β-unsaturated double bond. This kind of carbon–carbon bond weakly enhanced the corresponding antifungal activity of alcohols. Among the compounds investigated, (*E*)-2-nonenal had the highest degree of antifungal activity. Results indicated that, at the quantities investigated, C_9 aldehydes and ketones were generally more potent than their C_6 counterparts as antifungal agents vs *A. alternata*. One of the active α,β-unsaturated agents investigated, (*E*)-2-hexenal, was tested for mutagenicity using a volatile compound modification of the Ames test. No mutagenicity under the test conditions was detected for (*E*)-2-hexenal, a compound that is generally considered the most abundant volatile that plants emit as a response to wounding.

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LITERATURE CITED

Eder, E.; Deininger, C.; Neudecker, T.; Deininger, D. Mutagenicity of β-Alkyl Substituted Acrolein Congeners in the *S. typhimurium* Strain TA 100 and Genotoxicity Testing in the SOS Chromotest. *Environ. Mol. Mutagen.* **1992**, *19*, 338–345.

Einot, I.; Gabriel, K. R. A Study of the Powers of General Methods of Multiple Comparisons. *J. Am. Stat. Assoc.* **1975**, *70*, 574–583.

Feron, V. J.; Til, H. P.; de Vrijer, F.; Woutersen, R. A.; Cassee, F. R.; van Bladeren, P. J. Aldehydes: Occurrence, Carcinogenic Potential, Mechanism of Action and Risk Assessment. *Mutat. Res.* **1991**, *259*, 363–385.

French, R. C. Volatile Chemical Germination Stimulators of Rust and Other Fungal Spores. *Mycologia* **1992**, *84*, 277–288.

Gueldner, R. C.; Wilson, D. M.; Heidt, A. R. Volatile Compounds Inhibiting *A. flavus*. *J. Agric. Food Chem.* **1985**, *33*, 411–413.

Hamilton-Kemp, T. R.; McCracken, C. T., Jr.; Loughrin, J. H.; Andersen, R. A.; Hildebrand, D. F. Effects of Some Natural Volatile Compounds on the Pathogenic Fungi *Alternaria alternata* and *Botrytis cinerea*. *J. Chem. Ecol.* **1992**, *18*, 1083–1091.

Harman, G. E.; Mattick, L. R.; Nash, G.; Nedrow, B. L. Stimulation of Fungal Spore Germination and Inhibition of Sporulation in Fungal Vegetative Thalli by Fatty Acids and their Volatile Peroxidation Products. *Can. J. Bot.* **1980**, *58*, 1541–1547.

Hildebrand, D. F. Lipoxygenases. *Physiol. Plant.* **1989**, *76*, 249–253.

Iriye, R.; Aruga, R.; Toya, T.; Makino, J.; Dot, Y.; Handa, S.; Tanaka, H. Studies on Augmenting the Growth-Inhibition Activity of α,β-Unsaturated Aldehydes and Ketones Against *R. gracilis*. *Agric. Biol. Chem.* **1988**, *52*, 997–1001.

Lyr, H.; Banasiak, L. Alkenals, Volatile Defense Substances in Plants, their Properties and Activities. *Acta Phytopathol. Acad. Sci. Hung.* **1983**, *18*, 3–12.

Major, R. T.; Marchini, P.; Sproston, T. Isolation from *G. biloba L.* of an Inhibitor of Fungus Growth. *J. Biol. Chem.* **1960**, *235*, 3298–3299.

Marnett, L. J.; Hurd, H. K.; Hollstein, M. C.; Levin, D. E.; Esterbauer, H.; Ames, B. N. Naturally Occurring Carbonyl Compounds are Mutagens in *Salmonella* Tester Strain TA104. *Mutat. Res.* **1985**, *148*, 25–34.

Maron, D. M.; Ames, B. N. Revised Methods for the *Salmonella* Mutagenicity Test. *Mutat. Res.* **1983**, *113*, 173–215.

Maruzzella, J. C.; Chiaramonte, J. S.; Garofalo, M. M. Effects of Vapors of Aromatic Chemicals on Fungi. *J. Pharm. Sci.* **1961**, *50*, 665–668.

Nandi, B. Effect of Some Volatile Aldehydes, Ketones, Esters and Terpenoids on Growth and Development of Fungi Associated with Wheat Grains in the Field and in Storage. *Z. Pflanzenkrankh. Pflanzenschutz* **1977**, *84*, 114–128.

Shauenstein, E.; Esterbauer, H.; Zollner, H. α,β-Unsaturated Aldehydes. In *Aldehydes in biological systems*; Lagnado, J. R., Ed.; Pion: London, 1977; translated by P. H. Gore.

Simmon, V. F. Applications of the *Salmonella*/Microsome Assay. In *Short-term tests for chemical carcinogens*; Stich, H. F., San, R, H. C., Eds.; Springer: New York, 1981; pp 120–126.

Simmon, V. F.; Kauhanen, K.; Tardiff, R. G. Mutagenic Activity of Chemicals Identified in Drinking Water. In *Progress in genetic toxicology*; Scott, D., Bridges, B. A., Sobels, F. H., Eds.; Elsevier/North-Holland: Amsterdam, 1977; pp 249–258.

Spurr, H. W., Jr. An Efficient Method for Producing and Studying Tobacco Brown-Spot Disease in the Laboratory. *Tob. Sci.* **1973**, *17*, 145–148.

Urbasch, I. Production of C6-Wound Gases by Plants and the Effect on Some Phytopathogenic Fungi. *Z. Naturforsch.* **1984**, *39*, 1003–1007.

Winberry, W. T., Jr.; Murphy, N. T.; Riggen, R. M. Method for the Determination of Volatile Compounds in Ambient Air using Tenax Absorption and Gas Chromatography/Mass Spectrometry (GC/MS); EPA/600/4-89/017; U.S. Environmental Protection Agency, Research Triangle Park, NC, 1988.

Witz, G. Biological Interactions of α,β-Unsaturated Aldehydes. *Free Radical Biol. Med.* **1989**, *7*, 333–349.

Zeringue, H. J., Jr.; McCormick, S. P. Relationships between Cotton Leaf-Derived Volatiles and Growth of *Aspergillus flavus*. *J. Am. Oil Chem. Soc.* **1989**, *66*, 581–85.

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