# Effects of Six-Carbon Aldehydes and Alcohols on Bacterial Proliferation†

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Lipoxygenase/hydroperoxide lyase generated compounds are thought to be important in plant defense. The effects of volatile compounds from this pathway on the proliferation of *Escherichia coli* TB1, *Pseudomonas syringae* pv. *tabaci*, and *Pseudomonas syringae* pv. *angulata* were evaluated. The vapor-phase concentrations of compounds in the bioassay system were estimated by gas chromatography. At the highest concentrations tested, the $C_6$ aldehyde (*E*)-2-hexenal completely inhibited proliferation of both *P. syringae* pathovars (570 μg/L of air) and *E. coli* (930 μg/L of air). Similarly, the $C_6$ alcohol (*E*)-2-hexen-1-ol prevented proliferation of *P. syringae* pathovars (1100 μg/L of air) and *E. coli* (2300 μg/L of air). Among the bacteria tested, one isolate of *P. syringae* pv. *angulata* was the most sensitive to a lipoxygenase pathway volatile, exhibiting decreased proliferation after exposure to (*E*)-2-hexenal (40 μg/L of air). The unsaturated volatiles, (*E*)-2-hexenal and (*E*)-2-hexen-1-ol, exhibited a greater inhibitory effect than the saturated volatiles, hexanal and 1-hexanol. The responses to the volatile compounds observed for *E. coli* TB1 and *P. syringae* pv. *tabaci* were similar and differed somewhat from that of *P. syringae* pv. *angulata*.

## INTRODUCTION

$C_6$ aldehydes and $C_6$ alcohols are major volatile compounds produced by green leaves through the lipoxygenase/hydroperoxide lyase pathway (Hatanaka et al., 1987; Hildebrand, 1989). Fatty acids containing (*Z*),(*Z*)-1,4-pentadiene moieties such as linoleic (C18:2) and linolenic acid (C18:3), two major lipoxygenase (LOX, linoleate oxygen oxidoreductase, EC 1.13.11.12) substrates in higher plants, are converted by LOX to fatty acid hydroperoxides. The hydroperoxides of linoleic or linolenic acid are further converted by hydroperoxide lyase into hexanal or (*Z*)-3-hexenal, which isomerizes into the more stable (*E*)-2-hexenal. These aldehydes can be reduced to the corresponding alcohols by alcohol dehydrogenase (Hatanaka et al., 1987; Vick and Zimmerman, 1987a,b).

Volatile lipid peroxidation products from the LOX pathway are important for the organoleptic quality of certain plant-derived foods (Matoba et al., 1985). Accumulating evidence suggests that they may also be important for plant defense. Several in vitro studies have shown that volatile aldehydes and alcohols inhibit the growth of fungal species as determined by measurement of hyphal growth, dry matter accumulation, and/or germination of spores (Major et al., 1960; Nyman, 1969; Nandi, 1977; Urbasch, 1984; Gueldner et al., 1985; Zeringue and McCormick, 1989; Hamilton-Kemp et al., 1992). We have shown that these compounds produced by crushed foliage inhibited pollen germination (Hamilton-Kemp et al., 1991). The compounds were also shown to be inhibitory or toxic to some arthropods including mites and aphids (Lyr and Banasiak, 1983; our unpublished data). However, there are only a few studies on the effects of volatile compounds on bacteria (Surender et al., 1988; Együd, 1967; Együd and Szent-Györgi, 1966) and a protozoan (Schildknecht and Rauch, 1961). In studies with bacteria, the chemicals tested were added into cultures or were assayed by exposing broth cultures to compounds in the vapor phase. Information about the amounts of compounds in the vapor phase versus the effectiveness of these levels in inhibiting the growth of bacteria was not reported. No studies were found on the effects of volatile $C_6$ aldehydes and $C_6$ alcohols on bacterial pathogens of plants.

The objective of the present study was to evaluate the effects of vapor-phase $C_6$ aldehydes and $C_6$ alcohols on the proliferation of bacteria including plant pathogens. In our experiments, the bacteria were exposed to $C_6$ aldehydes and $C_6$ alcohols in the vapor phase, the quantities of which were measured by direct sampling from the headspace of the bioassay dishes and analysis with gas chromatography. The vapor-phase exposure approach was used since earlier studies showed that volatiles emitted from crushed leaves diffused through the air and inhibited pollen germination and fungal hyphal growth and that lipoxygenase pathway products contributed to the inhibition. Bacterial proliferation in the presence of the test volatiles as well as in the controls was determined by serial dilution and plating. In addition, the inhibition produced by $C_6$ volatiles with α,β unsaturation [(*E*)-2-hexenal and (*E*)-2-hexen-1-ol] was compared to that produced by the saturated volatiles (hexanal and 1-hexanol).

## EXPERIMENTAL PROCEDURES

**Bacteria.** Three bacterial strains, *Escherichia coli* TB1, *Pseudomonas syringae* pv. *tabaci* Deall (Shoemaker, 1991), and *Pseudomonas syringae* pv. *angulata* Deall (Shoemaker, 1991), were chosen for the present studies.

*E. coli* TB1 is a common laboratory bacterial strain used in molecular biology (Viera and Messing, 1982). A colony of *E. coli* TB1 was placed into 3 mL of LB broth [10 g of tryptone, 5 g of

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yeast extract (both from Difco Laboratories, Detroit, MI), and 10 g of sodium chloride/L of water] and cultured overnight at 37 °C.

*P. syringae* pv. *tabaci* and *P. syringae* pv. *angulata*, which cause the wildfire and angular leaf spot diseases, respectively, in tobacco (Shoemaker, 1991), were isolated from infected leaves that had been dried and stored at −20 °C. A few pieces of the leaves were placed into a test tube containing 5 mL of sterile water, soaked for 2–5 min, and then shaken for 2–3 min. Serial dilutions were prepared and streaked onto Bacto nutrient agar plates (Difco Laboratories, 23 g/L of water) and cultured at 31 °C for 2–3 days. *P. syringae* pv. *tabaci* or *P. syringae* pv. *angulata* colonies from the plates were placed into vials containing 5 mL of sterile water, vortexed, and stored at 4 °C. From the stocks, 10 μL of *P. syringe* pv. *tabaci* or *P. syringe* pv. *angulata* was placed into vials with 1 mL of Bacto nutrient broth (8 g/L of water) and maintained at 31 °C for 20–24 h to obtain stationary-phase cultures.

**Reagents.** $C_6$ aldehydes [(*E*)-2-hexenal and hexanal] and $C_6$ alcohols [(*E*)-2-hexen-1-ol and 1-hexanol] were purchased from Aldrich Chemical Co., Milwaukee, WI; additional chemicals were obtained from Sigma Chemical Co., St. Louis, MO. The following solutions were prepared in 1,2-propanediol (w/w): (*E*)-2-hexenal and hexanal at 10, 1, and 0.1% and (*E*)-2-hexen-1-ol and 1-hexanol at 10 and 1%. The alcohols were also tested neat. The reasons for choosing these concentrations were, first, to obtain similar amounts of aldehydes and alcohols in the vapor phase of the bioassay dishes and, second, the level of (*E*)-2-hexenal in the vapor phase from a 1% solution was close to that of naturally occurring (*E*)-2-hexenal released from crushed tomato leaves (Hamilton-Kemp et al., 1991).

**Bioassay.** All glassware and plastic tubes were autoclaved before use. The bioassay procedures (Hamilton-Kemp et al., 1992) were carried out under sterile conditions as follows:

First, 5 μL of overnight broth cultures of *E. coli* TB1 and *P. syringae* pv. *tabaci* and 6 μL of *P. syringae* pv. *angulata* were placed onto the top surface of an approximately 1-$cm^3$ block of LB agar (*E. coli*) or nutrient agar (*Pseudomonas*). The agar block had been placed in the center of a 5-cm glass Petri dish contained within a 9-cm glass Petri dish (120-mL volume). Second, 1 mL of sterile water was placed into a 1 cm diameter glass cup, and 10 μL of a solution of test compound or neat compound (as described above) was placed onto the water. The cover of the 9-cm Petri dish was replaced, and its outside edge was quickly wrapped with Parafilm to seal the dish. The assay dishes were placed into an incubator at 31 (*Pseudomonas*) or 37 °C (*E. coli*) for a 22.5-h exposure period. Control dishes were set up the same way as described above except 10 μL of neat 1,2-propanediol was added to the 1 mL of sterile water.

After the exposure period, each bioassay dish was opened, the agar block with bacteria was placed into a tube with 10 mL of LB (*E. coli*) or nutrient broth (*Pseudomonas*), and the tubes were vortexed four times for 10 s each. The bacteria were then diluted 100000-fold, and 100 μL of the diluted bacterial suspension was spread evenly onto LB or nutrient agar plates, which were maintained at 37 (*E. coli*) or 31 °C (*Pseudomonas*) overnight.

**Measurement of Bacterial Proliferation.** The numbers of bacteria produced during the period of exposure to the volatile aldehydes and alcohols (and in controls) and subsequent incubation were determined by counting the colony-forming units (CFUs) on the LB or nutrient agar plates with a Darkfield Quebec colony counter (Spencer).

There were at least six replicates in two independent experiments for each level of aldehyde and alcohol tested. Data were analyzed using analysis of variance and an LSD procedure at $p = 0.05$.

**Quantification of $C_6$ Aldehyde and $C_6$ Alcohol Levels in the Headspace.** The assay dishes were set up the same way as in the bioassay procedure except that no bacteria were added onto the agar block and the top cover of each 9-cm dish contained a hole fitted with a rubber septum. After an assay dish was maintained in an incubator at 31 (*Pseudomonas*) or 37 °C (*E. coli*) for 4 h to permit vapor-phase equilibration with solution, a gastight syringe was used to withdraw a 250-μL vapor sample from the headspace of the dish. This sample was injected into a Varian 3700 GC equipped with a 0.53 mm × 30 m DB-Wax [poly(ethylene glycol)] fused silica column. The operating conditions were as follows: inlet, 220 °C; column oven, 50 °C for 5 min, programmed at 3 °C/min to 150 °C; flame ionization detector, 240 °C; helium, 6 mL/min.

All measurements for each volatile at the three test concentrations were repeated at least six times by sampling from separate assay dishes.

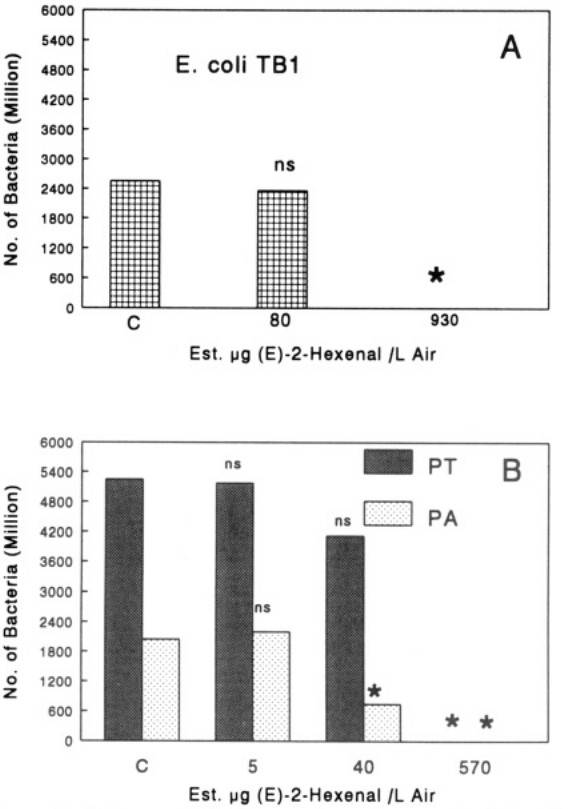


**Figure 1.** Effects of (*E*)-2-hexenal on the growth of (A) *E. coli* TB1 and (B) *P. syringae* pv. *tabaci* (PT) and *P. syringae* pv. *angulata* (PA). C, control, 1,2-propanediol without addition of the tested volatile compound; ns, the number of bacteria in the presence of the test compound at a given level was not significantly different from that in the control; *, significant differences between the number of bacteria exposed to the test compound and the control. Least significant difference (LSD) for *E. coli* = 930 million, for PT = 2280 million, and for PA = 740 million.

## RESULTS AND DISCUSSION

**Effects of $C_6$ Aldehydes.** The effects of the unsaturated and the saturated $C_6$ aldehydes, (*E*)-2-hexenal and hexanal, on proliferation of three bacterial strains are presented in Figures 1 and 2. (*E*)-2-Hexenal at a concentration of 80 μg/L of air had no significant effect on the proliferatioin of *E. coli* TB1 following the end of the exposure period (Figure 1). However, 930 μg/L of (*E*)-2-hexenal completely inhibited cell division and was considered toxic as no proliferation of *E. coli* occurred following the exposure period and a subsequent incubation of up to 2 days. (*E*)-2-Hexenal in the vapor phase at 5 μg/L of air had no significant inhibitory effect on the growth of either *P. syringae* pv. *tabaci* or *P. syringae* pv. *angulata*. An intermediate concentration (40 μg/L) of this compound had a significant inhibitory effect on *P. syringae* pv. *angulata* but not on *P. syringae* pv. *tabaci*. The highest concentration, 570 μg/L, completely inhibited proliferation of both bacteria (Figure 1).

Hexanal in the vapor phase at 200 μg/L of air had no significant effect on the proliferation of *E. coli* TB1; however, 2800 μg/L significantly inhibited its proliferation

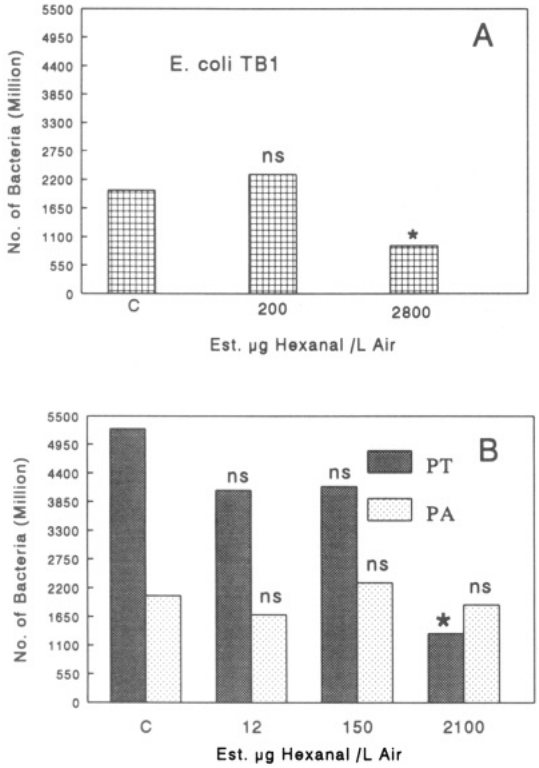


**Figure 2.** Effects of hexanal on the growth of (A) *E. coli* TB1 and (B) *P. syringae* pv. *tabaci* (PT) and *P. syringae* pv. *angulata* (PA). C, ns, and * are the same as in Figure 1. LSD for *E. coli* = 1000 million, for PT = 2280 million, and for PA = 740 million.

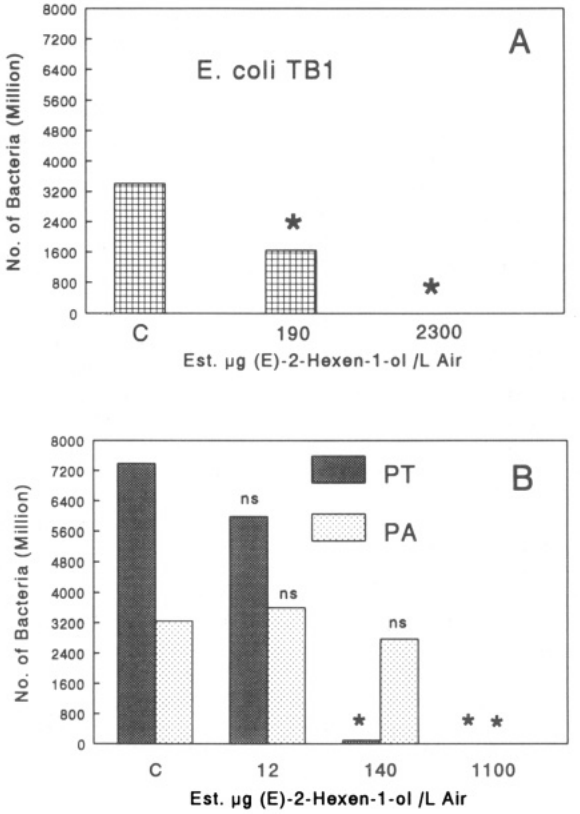


**Figure 3.** Effects of (*E*)-2-hexen-1-ol on the growth of (A) *E. coli* TB1 and (B) *P. syringae* pv. *tabaci* (PT) and *P. syringae* pv. *angulata* (PA) C, ns, and * are the same as in Figure 1. LSD for *E. coli* = 440 million, for PT = 2200 million, and for PA = 2400 million.

(Figure 2). The saturated aldehyde at 12 or 150 µg/L of air had no effect on either *P. syringae* pv. *tabaci* or *P. syringae* pv. *angulata*. However, hexanal at 2100 µg/L significantly inhibited growth of *P. syringae* pv. *tabaci* but did not affect the growth of *P. syringae* pv. *angulata* (Figure 2).

Surender et al. (1988) reported that large amounts of 2-hexenal and 2-heptenal tested in the vapor phase were toxic to *E. coli* in liquid culture. However, no measurement of the vapor-phase concentration of the compounds was reported. (*E*)-2-Hexenal and hexanal tested as volatiles have been shown to be inhibitory to the germination and growth of several pathogenic fungi (Nandi, 1977; Zeringue and McCormick, 1989; Hamilton-Kemp et al., 1992) and pollen (Hamilton-Kemp et al., 1991). (*E*)-2-Hexenal was markedly more active than hexanal in our studies with pollen and fungi, with inhibition of the growth generally occurring in the concentration range of 20 µg (*E*)-2-hexenal/L of air. They can also inhibit germination of seeds including some important crop and weed species (French et al., 1979; Bradow et al., 1990; Gardner et al., 1990). Aldehydes of lower or higher carbon numbers such as formaldehyde and acetaldehyde or 2-nonenal were also shown to be inhibitory to the growth of *E. coli* or fungi when added as liquids to the culture medium (Együd, 1967; Nandi, 1977). In contrast to these findings, a number of studies on hyphal growth and spore germination of fungi have shown that several volatile compounds, including saturated aldehydes such as nonanal, exhibit stimulatory effects at low concentrations [see reviews by Fries (1973) and French (1985)]. However, it is difficult to compare these results to the present studies since the amounts of volatiles in the vapor phase were not available or the compounds were incorporated directly into agar for testing.

In our study, most concentrations of the headspace volatiles that showed inhibitory effects on bacterial growth exceeded the levels which were obtained from wounded plant leaves. However, the level of (*E*)-2-hexenal showing inhibition of *P. syringae* pv. *angulata* was estimated at 40 µg/L of air, which was approximately 2 times the level that was measured in the headspace from wounded tomato leaves (Hamilton-Kemp et al., 1991). The actual quantities of volatiles produced by leaves are expected to be higher than the amounts measured since a portion of compounds such as the aldehydes would not be expected to be released due in part to reactivity with cellular constituents (discussed below).

In our preliminary screening experiments, the volatile (*Z*)-3-hexenyl acetate, another naturally occurring product derived from the LOX pathway, had no significant effect on the proliferation of *E. coli* TB1 (data not shown). However, a similar compound, (*E*)-2-hexenyl acetate, was reported to inhibit growth of two bacterial species, *E. coli* and *Streptococcus albus* (Surender et al., 1988). Volatiles from wounded tomato leaves [e.g., (*E*)-2-hexenal at approximately 20 µg/L of air] were shown to inhibit pollen germination and growth of two fungal species in vitro (Hamilton-Kemp et al., 1991, 1992), but in our assay studies, wounded leaves did not show any significant effect on *E. coli* TB1. In addition, tests with the solvent 1,2-propanediol in the controls indicated that this compound did not affect the bacteria (data not shown).

**Effects of $C_6$ Alcohols.** (*E*)-2-Hexen-1-ol in the vapor phase at 190 µg/L of air significantly inhibited proliferation of *E. coli* TB1, and 2300 µg/L completely inhibited growth (Figure 3). However, (*E*)-2-hexen-1-ol at 12 µg/L had no significant effect on *P. syringae* pv. *tabaci*, while concentrations of 140 µg/L of air significantly inhibited growth of *P. syringae* pv. *tabaci* but had no effect on *P. syringae* pv. *angulata*. (*E*)-2-Hexen-1-ol at 1100 µg/L of air was toxic to both *Pseudomonas* bacteria.

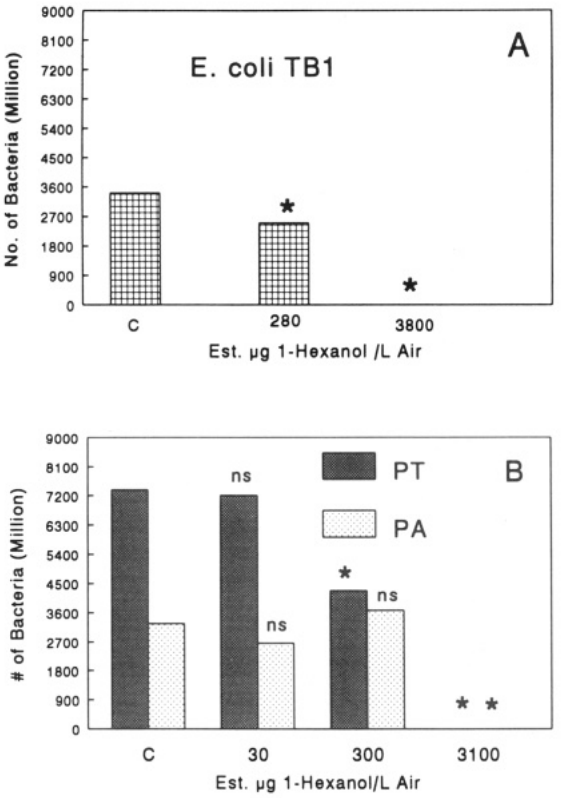


**Figure 4.** Effects of 1-hexanol on the growth of (A) *E. coli* TB1 and (B) *P. syringae* pv. *tabaci* (PT) and *P. syringae* pv. *angulata* (PA). C, ns, and * are the same as in Figure 1. LSD for *E. coli* = 440 million, for PT = 2200 million, and for PA = 2400 million.

Figure 4 shows the effects of 1-hexanol on the proliferation of the three bacterial strains. 1-Hexanol in the vapor phase at 280 µg/L significantly inhibited proliferation of *E. coli* TB1, whereas 3800 µg/L inhibited proliferation completely. Tests with *P. syringe* pv. *tabaci* showed that 1-hexanol at 30 µg/L of air had no significant effect on proliferation. 1-Hexanol at 310 µg/L inhibited growth of *P. syringe* pv. *tabaci* but had no effect on *P. syringae* pv. *angulata*. The saturated alcohol at 3100 µg/L completely inhibited the proliferation of both pathovars.

(*E*)-2-Hexen-1-ol or 1-hexanol was reported to be toxic to insects (Lyr and Banasiak, 1983), to inhibit the growth of fungal species (Gueldner et al., 1985), and to inhibit the germination of pollen (Hamilton-Kemp et al., 1991) and seeds (Bradow et al., 1988). The mechanisms of alcohol toxicity is not clear. The mechanism hypothesized for the toxicity of ethanol in plant tissues is that ethanol changes the permeability of cell membranes, thus causing leakage of cellular components and influencing transport of inorganic compounds (Jackson et al., 1982). The greater toxicity of unsaturated alcohols could be due to the conversion to the more toxic unsaturated aldehydes (see next section) by alcohol dehydrogenase as described by Perata and Alpi (1991).

**Volatile Compounds with α,β Unsaturation Elicit Stronger Growth Inhibition.** As described above, compounds with α,β unsaturation increased inhibition of bacterial proliferation relative to that observed with the saturated compounds. This is consistent with the greater effects of (*E*)-2-hexenal and (*E*)-2-hexen-1-ol on the inhibition of pollen germination compared to those of hexanal and 1-hexanol (Hamilton-Kemp et al., 1991). Also, (*E*)-2-hexenal exhibited greater inhibitory effects than hexanal in assays involving seed germination (Bradow and Connick, 1990; Gardner et al., 1990) and the growth of fungi (Urbasch, 1984; Zeringue and McCormick, 1989; Hamilton-Kemp et al., 1992).

The inhibition or toxic effect of α,β-unsaturated aldehydes is thought to be due to reaction of the compounds with the sulfhydryl moiety or cysteine residues or formation of Schiff bases with amino groups of peptides and proteins (Schauenstein et al., 1977). This interaction might change the conformation of some membrane-bound proteins, altering membrane structures as supported by electron microscopic observations of morphological changes in mitochondrial and nuclear membranes of *Mucor mucedo* hyphae (Lyr and Banasiak, 1983). Alternatively, the interaction might inactivate some enzymes that are necessary for gene expression, cellular metabolism, or cell growth and division.

α-Ketoaldehydes, which have a C=O adjacent to the aldehyde group (compared to C=C of α,β-unsaturated aldehydes), have been reported to inhibit the growth of *E. coli* (Együd, 1967). Együd and Szent-Györgi (1966) also obtained evidence that α-ketoaldehydes were toxic to *E. coli* due to reaction of these compounds with the sulfhydryl group of cysteine.

**Differential Bacterial Responses.** In our study, three different bacterial strains were chosen for the assay. *E. coli* TB1 is a modified coliform bacterium widely used in molecular biological studies (Viera and Messing, 1982). *P. syringae* pv. *tabaci* and *P. syringae* pv. *angulata*, the taxonomy of which is still unresolved, are two plant pathogenic bacteria. These pathovars cannot be distinguished by morphological, cultural, biochemical, or serological tests except for the production of the nonspecific toxin, tabtoxin, and the presence of a yellow halo around the tobacco leaf spots for *P. syringae* pv. *tabaci* (Deall and Cole, 1986; Shoemaker, 1991).

It is interesting that, under our assay conditions, the strains of *E. coli* TB1 and *P. syringae* pv. *tabaci* responded to the test volatiles in a similar manner and the response differed from that of *P. syringae* pv. *angulata*. (*E*)-2-Hexenal in the vapor phase at the intermediate levels had no significant effects on either *E. coli* TB1 or *P. syringae* pv. *tabaci* but significantly decreased the proliferation of *P. syringae* pv. *angulata*. Hexanal at the highest levels significantly decreased the proliferation of both *E. coli* TB1 and *P. syringae* pv. *tabaci* but not that of *P. syringae* pv. *angulata*. At the intermediate concentrations tested, (*E*)-2-hexen-1-ol significantly decreased the proliferation of *E. coli* TB1 and *P. syringae* pv. *tabaci* but showed no significant influence on *P. syringae* pv. *angulata*. Similarly, 1-hexanol at the intermediate level significantly decreased the proliferation of *E. coli* TB1 and *P. syringae* pv. *tabaci* but showed no significant influence on *P. syringae* pv. *angulata*.

The mechanism by which bacteria responded differently to the authentic chemicals is not known. Surender et al. (1988) reported that *E. coli* and *Streptococcus albus* responded similarly to 11 compounds in bioassays. However, 2-octenal was found to inhibit growth of *S. albus* more than that of *E. coli*. In a study of the metabolism of 2-hexenal by the fungus *Botrytis cinerea*, Urbasch (1987) reported differences among five isolates. Isolates that were exclusively mycelium-forming metabolized 2-hexenal to 2-hexen-1-ol, whereas spore-forming isolates converted 2-hexenal to 1-hexanol.

This paper provides a foundation for the evaluatioin of the present and potential role of LOX pathway products in the plant pest defense process. Whether plant tissues can produce levels of volatile compounds which are of significance in defense against pathogens is a very complex

question to address. Once $C_6$ aldehydes are formed in plant cells, part would be expected to react with protein and other cellular components; part escapes into gaseous intercellular spaces, and some is emitted from the leaf surface. In natural situations, when a pathogen attacks plants, the portion of $C_6$ aldehyde that is most important for the inhibition of pathogen spread should be the part that is in the intercellular space and near the leaf surface. However, it is very difficult to measure such levels of $C_6$ aldehydes with our present techniques. It may be possible for us to use genetic transformation approaches to obtain transgenic plants with increased disease resistance, by increasing production of these compounds to levels effective in inhibiting growth of pathogens. We estimate that (*E*)-2-hexenal produced in plant tissues at 1 nmol/g would significantly reduce growth of some pathogenic bacteria. Plant leaves generally have >3 μmol of linolenic acid/g, which is the precursor of (*E*)-2-hexenal. Therefore, less than 0.1% of linolenic acid present in plant leaves would need to be converted by lipoxygenase/lyase into (*E*)-2-hexenal.

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