Volatile Products of the Lipoxygenase Pathway Evolved from *Phaseolus vulgaris* (L.) Leaves Inoculated with *Pseudomonas syringae* pv *phaseolicola*1

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...plants and found the C₆ volatile-forming system was present in all the plants tested but with varying degrees of constitutive activity in different species. The enzymic breakdown of linolenic acid produces a range of products and intermediates, many of which contribute to characteristic odors of cut or damaged plant tissue (Hatanaka et al., 1987; Siedow, 1991). The biological activity of many of these substances is poorly understood (Vick and Zimmerman, 1987a; Siedow, 1991) but Vick and Zimmerman (1984) noted that some of the products had structures similar to those of the regulatory eicosanoids of animals and suggested that “octadecanoids” (i.e. C₁₅ lipoygenase-pathway products derived from linoleic or linolenic acid) could fulfill similar roles in plants. Thus, it has been suggested that lipoxygenase activity might produce signal molecules important in host-pathogen interactions (Anderson 1989; Slusarenko et al., 1991; Farmer and Ryan, 1992). In this work, we explore the possibility that lipid-derived volatiles, produced ultimately as a result of lipoxygenase activity, may also have a more direct effect on the pathogens themselves.

Depending on the source of the enzyme, lipoxygenase activity on linolenic acid will produce either 9- or 13-hydroperoxylinolenic acid or a mixture of both. In bean, the 13-hydroperoxide is the major product of lipoxygenase activity (Matthew and Galliard, 1978). Figure 1 shows how a hydroperoxide lyase can then catalyze the breakdown of 13-hydroperoxylinolenic acid to a C₁₃ product, cis-3-hexenal, and a C₁₂ product, 12-oxo-cis-9-dodecenoic acid. In further reactions, cis-3-hexenal can form cis-3-hexenal, trans-2-hexenal, trans-3-hexenol, or trans-2-hexenal (Fig. 1). The C₁₂ product, 12-oxo-cis-9-dodecenoic acid, is a precursor of the plant wound hormone traumatic acid (Strong and Kruitwagen, 1967; Vick and Zimmerman, 1987a; Anderson, 1989; Siedow, 1991). The 9-hydroperoxide of linolenic acid, when present, is converted in bean to trans-2,cis-6-nonadienal by a corresponding lyase activity (Matthew and Galliard, 1978).

**trans-2-Hexenal** (C₆H₁₀O) is a six-carbon α,β-unsaturated aldehyde and is virtually immiscible with water (CRC Handbook of Chemistry and Physics, 1989–1990). Although it has been suggested that *trans*-2-hexenal may be involved in the defense mechanism of plants to pathogens (Lyr and Basaśniak, 1983; Vick and Zimmerman, 1987a) it has not until now been demonstrated that *trans*-2-hexenal was produced in host-
pathogen interactions. *trans*-2-Hexenal was isolated from leaves of *Ginkgo biloba* and was reported to inhibit growth of the fungus *Monilinia fructicola* at a concentration of 300 ppm (Major et al., 1960). It was suggested that *trans*-2-hexenal was involved in the ginkgo tree's remarkable resistance to pests and diseases. In various studies, *trans*-2-hexenal has also been shown to possess antifungal (Von Schildknecht and Rauch, 1961), antifungal, insecticidal, and acaricidal activity (Lyr and Basaniak, 1983). Recently, Zeringue and McCormick (1989) demonstrated that volatiles, including *trans*-2-hexenal, produced from wounded cotton leaves were able to inhibit growth of the saprophytic fungus *Aspergillus flavus* growing in liquid cultures. Matthew and Galliard (1978) proved that *trans*-2-hexenal and certain other products were formed from fatty acid hydroperoxides via the lipoxygenase pathway during wounding in *Phaseolus vulgaris* and showed the lyase and cleavage enzymes were present constitutively. We previously demonstrated an increase in lipoxygenase isozymes in beans undergoing a HR after inoculation with an avirulent race of the plant pathogenic bacterium *Pseudomonas syringae pv phaseolicola* but found no increase in activity in the susceptible response after inoculation with virulent bacteria (Croft et al., 1990). Fatty acid peroxidation was shown to be occurring in vivo during the HR by monitoring alkane gas production (ethane and pentane) from whole leaves (Croft, 1990; Croft et al., 1990). Ethane and pentane evolution has been widely used as a reliable index of in vivo lipid peroxidation (Konze and Elstner, 1978; Kunert and Tappel, 1983; Slater, 1984). Evidence for a role for lipoxygenase in plants during disease responses has accumulated in recent years (Kepler and Novacky, 1986, 1987; Ocampo et al., 1986; Yamamoto and Tani, 1986; Ohta et al., 1990), and elicitor-induced lipid peroxidation has been reported in bean (Rogers et al., 1988).

Substrates for lipoxygenase, such as linoleic and linolenic acids, are common constituents of plant membranes (Epton and Deverall, 1968; Hoppe and Heitefuss, 1974) but are not readily acted upon by the enzyme unless they are in the free acid form (Leshem, 1984). Polyunsaturated fatty acids are very susceptible to autoxidative processes in the free acid form but much less so when they are in situ in membranes (Galliard, 1978). Hoppe and Heitefuss (1974) established that linoleic and linolenic acids were among the most common fatty acids found in the lipid components of healthy *P. vulgaris* membranes, linolenic being more common than linoleic. Epton and Deverall (1968) calculated that linolenic acid...
was present in *P. vulgaris* leaves at a level of around 320 µg g⁻¹ of leaf tissue.

The presence of suitable substrates, the evidence of increases in lipoxygenase activity as a widespread plant disease-resistance response, and the constitutive presence of the other necessary enzymes indicate that the production of lipid-breakdown substances may be an important factor in disease resistance. In view of the antifungal and antiprotozoal activity of trans-2-hexenal reported in the literature (Major et al., 1960; von Schildknecht and Rauch, 1961), it seemed feasible that trans-2-hexenal and perhaps other fatty acid-derived substances produced in the plant were involved in the resistance of *P. vulgaris* to infection with *P. syringae pv phaseolicola*. Experiments were carried out to determine if any lipid-derived volatile substances were produced during the HR of *P. vulgaris* induced by *P. syringae pv phaseolicola* and to test the effect in vitro of cis-3-hexenol and trans-2-hexenal on *P. syringae pv phaseolicola*. Because traumatic acid is formed from the remaining moiety of linolenic acid when C₆ volatiles are produced, the effect of this substance on *P. syringae pv phaseolicola* was also investigated. The possible importance of volatile lipid-breakdown products in plant defense responses is discussed.

**MATERIALS AND METHODS**

**Plant and Bacterial Material**

Seeds of *Phaseolus vulgaris* (L.) cv Red Mexican UI3 were lightly scarified using fine glasspaper, allowed to imbibe tap water overnight, with several water changes, and planted individually in 2.5-inch plastic pots containing compost. The pots were transferred to growth cabinets with a 16-h photo-period at day and night temperatures of 25°C and 22°C, respectively. The primary leaves were ready for vacuum infiltration approximately 8 to 9 d after sowing.

*Pseudomonas syringae pv phaseolicola* (Burkholder) isolate 31A (race 1) and isolate 1301A (race 3), donated by Dr. J.D. Taylor, Institute of Horticultural Research, Wellesbourne, UK, were obtained from the culture collection of the Department of Applied Biology, University of Hull, England. Working cultures were routinely maintained on nutrient agar (Oxoid) supplemented with 1% (v/v) glycerol (NAG plates). After approximately 18 h of growth, cultures were centrifuged at 3000g for 1 to 2 min at 25°C. The day before inoculation, cells growing in NBG were used to inoculate a 25-mL culture of King's B broth (20% [w/v] peptone No. 3 [Difco], 2% [w/v] KH₂PO₄, 3H₂O, 1.5% [w/v] MgSO₄·7H₂O). After approximately 48 h of growth in NBG, cultures were centrifuged at 3000g for 10 min and the bacterial pellet resuspended gently in nutrient broth (Oxoid) containing 1% (v/v) glycerol (NBG) in a 25-mL Sterilin tube in an orbital shaking incubator at 150 cycles min⁻¹ at 25°C. The day before inoculation, cells growing in NBG were used to inoculate a 25-mL culture of King's B broth (20% [w/v] peptone No. 3 [Difco], 2% [w/v] KH₂PO₄, 3H₂O, 1.5% [w/v] MgSO₄·7H₂O). After approximately 18 h of growth, cultures were centrifuged at 3000g for 10 min and the bacterial pellet resuspended gently in inoculation buffer (1 buffer = 0.0014 M KH₂PO₄, 0.0025 M Na₃HPO₄, pH 7.00) and adjusted to an A₆₀₀ of 0.06, which dilution plate counts showed to contain approximately 1 × 10⁸ cfu mL⁻¹. Plants were vacuum infiltrated as previously described (Croft et al., 1990). The underside of the plants were dark green and water soaked after inoculation but regained normal appearance after 30 min in the growth cabinet. The inoculated leaf area was regularly 90% or more of the total. After inoculation, plants were returned to the growth chamber and placed under constant light.

**Bacterial Growth in Vivo**

Leaf discs (10, 1 cm in diameter) were cut from inoculated leaves at various times after inoculation and ground up in 5 mL of inoculation buffer with a mortar and pestle, and serial dilutions in inoculation buffer were plated out and incubated for 3 d at 25°C on nutrient agar (Oxoid) plates supplemented with 1% (v/v) glycerol to determine bacterial numbers. The data from three independent inoculations were pooled and each time point represents the data from three to six plants collectively.

**Effect of trans-2-Hexenal on Bean Plants**

Individual seedlings were placed in airtight polypropylene containers (13 cm high, enclosed volume 1 L). One proprietary cotton bud (Johnson & Johnson) was placed in the compost in each pot, and either 25 µL of ethanol or 5, 10, or 25 µL of trans-2-hexenal dissolved in ethanol was pipetted onto it. The pots were sealed and replaced in the growth chamber. After 2 d, leaf tissue was extracted and analyzed for phytoalexins as described by Slusarenko et al. (1989). The experiments were performed twice with two seedlings each time.

**Collection of Plant Volatiles and GC-MS Analysis**

The method used was that of Jüttner (1988) modified for the analysis of emission products. The plant hypocotyl was cut with a scalpel approximately 1 to 2 cm up from soil level and the base wrapped in aluminum foil to prevent water loss from the cut surface. The plant was then placed in an airtight glass container with two ports. The exit port was attached to a collection cartridge containing 150 mg of Tenax TA sorbent (40–60 mesh) and then by a Luer lock via copper tubing to a stainless steel pump and back via the inlet port to the glass vessel. Air in the enclosed system was circulated at a flow rate of 1.2 L min⁻¹ for 30 min over the Tenax sorbent. Two 40-W light bulbs were placed near to the exit port over the collection cartridge and Tenax bed to prevent water condensation. Once collection of volatiles was complete, the cartridge was sealed in an air-tight glass-stoppered vessel until it was analyzed by GC-MS (Hewlett-Packard 5970A).

To desorb the collected volatiles, the Tenax cartridge was placed in a movable heating block (40-W capacity) and fixed to a helium gas supply with a flow rate of 44 mL min⁻¹ while the Luer end of the column was fixed with a side-port needle and introduced through the GC injection port septum. The internal carrier gas supply for the GC was switched off and the gas supply passing over the Tenax collection cartridge allowed to replace it. The oven was cooled to 0°C to trap the released volatiles and the Tenax cartridge was heated to 270°C over 2 min and maintained at this temperature for 2 min.
Preparation of Hexenal-Saturated Buffer and Quantification by GLC

Approximately 25 μL of trans-2-hexenal (Sigma) was added to 200 μL of inoculation buffer, vortexed, and allowed to equilibrate overnight at room temperature. The undissolved trans-2-hexenal was visible as an oily layer on the surface, and the saturated buffer below the trans-2-hexenal was removed using a syringe and needle. Quantification of trans-2-hexenal dissolved in the buffer was by GC according to the method of Galliard et al. (1986). Aliquots of up to 10 μL of the trans-2-hexenal-saturated buffer were analyzed on a Pye Unicam GCD gas chromatograph containing a 1.5 m x 4 mm column packed with 10% Carbowax, chromosorb W 80/100 mesh, acid washed, and dimethyldichlorosilane treated. The column temperature was 110°C, and the detection and injection temperatures were 180°C. The carrier gas was nitrogen at a flow rate of 30 mL min⁻¹. trans-2-Hexenal is readily soluble in pentane (Matthew and Galliard, 1978), so a series of standard solutions was prepared using authentic trans-2-hexenal (Sigma) dissolved in pentane. Under the analytical conditions described, the retention time for the trans-2-hexenal standard was approximately 2 min 20 s, no other peaks being detected.

The undissolved trans-2-hexenal-saturated buffer was diluted to 70 were used in the selected ion monitoring mode to determine trans-2,cis-6-nonadienal quantitatively. Under the analytical conditions used, the detection limit for this compound was 8 ng.

Activity on Bacterial Growth of trans-2-Hexenal Spotted on Filter Discs

Approximately 10 μL of an overnight culture of P.s. pv. phaseolicola, grown in NBG medium, was centrifuged at 4000g in a Denley bench centrifuge for 10 min, and the pellet was resuspended in approximately 5 mL of I buffer. The optical density of the bacterial solution was adjusted to A₆₀₀ = 0.06. Approximately 150 μL of bacterial suspension was pipetted onto a fresh, dry NAG plate and evenly distributed with a glass spreader. The plate was covered and incubated for 4 h at 25°C to allow the agar surface to dry. Plates seeded in this way showed heavy growth of bacteria within 1 d when incubated at 25°C.

To test the effect of trans-2-hexenal on growth of bacteria on NAG plates, sterile 14 mm x 2 mm filter discs (Whatman) were placed centrally on the agar surface of previously seeded NAG plates. Different amounts of trans-2-hexenal or trans-2-hexenal-saturated I buffer were pipetted onto filter discs on Petri plates. trans-2-Hexenal was applied in a range from 75 to 1 μL. trans-2-Hexenal-saturated buffer was diluted to 50 μL with I buffer to give aliquots containing 167, 83.3, 16.5, 1.7, 0.53, or 0.03 nL of trans-2-hexenal. These aliquots were then spotted onto filter discs. Control plates were prepared by pipetting 50 μL of I buffer onto filter discs. Plates were then sealed with Nescofilm and incubated at 25°C overnight. The experiment was repeated four times.

Effect on Bacterial Growth of trans-2-Hexenal in Agar Plugs

Sterile 9-cm Petri dishes containing NAG to a depth of 5 mm had 10-mm diameter wells cut in the agar with a cork borer. Various amounts of trans-2-hexenal-saturated buffer were brought to 105 μL with 1 buffer and pipetted into the wells, then mixed with 150 μL of 45°C NAG medium by drawing back and forth with a pipette. A control with no trans-2-hexenal was also prepared. Once the agar plugs had set, 300 μL of P.s. pv. phaseolicola, adjusted with 1 buffer to A₆₀₀ = 0.1, was mixed with 5 mL of 45°C molten top agarose (0.4% agarose, 10 mM MgS0₄) then poured onto the agar plate. Incubation was overnight at 25°C. Another agarose overlay including a chromogenic substrate was used to distinguish between areas where bacteria had grown and where they had been killed, and was adapted from the TLC overlay method of Slusarenko et al. (1989). Molten agarose (5 mL) at 45°C was taken to 0.1% (w/v) with TTC (Sigma), mixed thoroughly, poured over the bacteria-seeded agarose surface, and incubated at room temperature. Color development began almost immediately and was complete by the next day. Areas where bacterial growth was inhibited were colorless, whereas areas of bacterial growth were pink due to the reduction of TTC to formazans by bacterial dehydrogenases. The experiment was repeated six times.

Effect of trans-2-Hexenal on Bacterial Growth in Liquid Cultures

Sterile 250-μL flasks containing 25 mL of NBG were inoculated with 2 mL of an overnight culture of P.s. pv. phaseolicola and 1 μL (0.35 mM), 5 μL (1.73 mM), or 25 μL (8.65 mM) of trans-2-hexenal was added per flask. Growth was compared with that in a control flask containing no trans-2-hexenal. The flasks were incubated in an orbital shaker at 25°C, 150 rpm, and changes in the A₆₀₀ of the flasks were measured over time. After 2.5 h of incubation, a
loopful of culture from each flask was streaked onto a NAG plate and the plates were incubated as before.

The experiment above was repeated using various quantities of trans-2-hexenal-saturated 1 buffer added to the bacterial cultures. The final concentrations of trans-2-hexenal in the flasks were 173, 86.4, 57.6, 28.8, and 5.8 μM. The experiment was repeated twice.

EM of trans-2-Hexenal-Treated Bacteria Growing in Liquid Culture

A 100-mL culture of P.s. pv phaseolicola with an A_{400} of 1.00 was made 2.2 mm with trans-2-hexenal and incubated at 25°C with shaking at 150 cycles min⁻¹. The A_{400} was measured every 15 min for the next 1.5 h, by which time it had dropped to approximately 0.8. A control flask was treated with 25 μL of sterile distilled water. Bacteria were collected by centrifugation at 4000g for 10 min and resuspended in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, and left for 1 h at room temperature. The bacteria were washed 3 times in 0.1 M phosphate buffer, pH 7.2, at room temperature for 5 min and collected by centrifugation. The final resuspension was in post-fixative 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer, pH 7.2, and samples were left at room temperature for 30 min before being centrifuged and washed four times for 5 min each time in 0.1 M phosphate buffer, pH 7.2. Dehydration of the samples was carried out in a graded ethanol series, with 5 min in each solution, up to absolute ethanol. Samples were embedded in an araldite epoxy mixture and polymerized at 70°C for 24 h. Sections were cut using glass knives on an ultramicrotome and mounted onto uncoated 300-mesh copper grids. Staining followed, in 2% (w/v) uranyl acetate for 30 min and lead citrate for 5 min. Sections were viewed on a JEOL 100 c electron microscope at 80 kV.

Effect of Traumatic Acid on Bacterial Growth in Agar Plugs

Sterile 6-cm Petri dishes were filled to a depth of 5 mm with NAG and allowed to dry, and 10-mm diameter wells were cut centrally. Because at the concentrations used the cis-3-hexenol (Sigma) was highly insoluble in the water-based agar, it was necessary to add ethanol and DMSO as solubilizing agents to final concentrations of 8% (v/v) and 36% (v/v), respectively. The cis-3-hexenol solution was taken to a total volume of 140 μL with water and then mixed with 140 μL of molten NAG at 45°C and pipetted into the central well of the agar plate and allowed to solidify. The volume of cis-3-hexenol added to the plates ranged from 1 to 15 μL. A control plate was prepared in the same way but with no cis-3-hexenol. The bacterial overlay and subsequent chromogenic overlay were used as described above for the trans-2-hexenal but were scaled down for the smaller size of the Petri dishes to 2.5 mL of each overlay solution. The experiment was repeated four times.

Effect of Traumatic Acid on Bacterial Growth in Liquid Cultures

Sterile 250-mL flasks containing 25 mL of NBC were inoculated with P.s. pv phaseolicola as above and traumatic acid (Sigma) was added to the flasks to final concentrations of 1.88, 0.94, and 0.47 mM. Because traumatic acid is very insoluble in aqueous solutions, it was first dissolved in DMSO and then added to the flasks; DMSO was also added to the control flask (no traumatic acid). The final concentration of DMSO in the culture medium was 2.9% (v/v), which had no apparent effect on bacterial growth in the control but kept the traumatic acid in solution. It was impossible to take traumatic acid to a concentration above 2 mM because of its insolubility and the undesirability of higher DMSO concentrations. The experiment was repeated twice.

RESULTS

Production of Lipid-Derived Volatiles by Inoculated Plants

Plants inoculated with either race 1 or race 3 of P.s. pv phaseolicola produced a range of volatiles (Fig. 2), and plants undergoing an HR produced the greatest amounts. Between 15 and 24 h after inoculation the average amounts of trans-2-hexenal and cis-3-hexenol produced by plants undergoing an HR were approximately 17 and 16 times higher, respectively, than the amounts produced by plants undergoing a susceptible response (Fig. 3, A and B). The identity of the volatiles was confirmed by GC-MS, and retention times and mass spectra were compared to those of authentic reference substances. The mass spectra of the substances putatively identified as trans-2-hexenal and cis-3-hexenol are shown in Figure 4, A and B. At 24 h after inoculation the HR, cis-3-hexenol ("leaf alcohol") was the major volatile detected and the second most abundant substance was cis-3-hexenal, which is the precursor of cis-3-hexenal (Fig. 1). Direct and indirect pathways can lead to trans-2-hexenal production from cis-3-hexenal, and, indeed, a large peak of trans-2-hexenal was detectable, as well as a smaller peak of trans-2-hexenol, the alcohol that is formed from reduction of trans-2-hexenal by alcohol dehydrogenase.

It is interesting that when plants showed good hypersensitive cell collapse (i.e. from 15–24 h after inoculation), the leaves emitted a distinctive odor due to volatile production, somewhat like dried tea. Indeed, cis-3-hexenol and trans-2-hexenal are mainly responsible for the "fresh" odor of green tea (Takeo, 1992). Buffer-inoculated plants, or plants undergoing a susceptible response at 24 h after inoculation, did not have this distinctive odor.

Effects of trans-2-Hexenal on Bean Plants

At the concentrations tested, bean plants showed loss of turgor by 48 h after beginning treatment. By this time, plants that received 25 μL L⁻¹ trans-2-hexenal had collapsed completely and dried out. At the lower concentrations, plants were flaccid and epinastic. TLC of ethanol extracts of leaf tissue showed that trans-2-hexenal-treated plants had suffered a reduction in photosynthetic pigments compared with the controls. No isoflavonoid phytoalexins were detected.

Effects of trans-2-Hexenal on Bacteria

As little as 1 μL of trans-2-hexenal spotted onto a filter disc prevented all bacterial growth on 9-cm diameter NAG plates.
Figure 3. A, Liberation of trans-2-hexenal by inoculated plants and B, liberation of cis-3-hexenol. Each data point shows the amount liberated by a single plant over a 30-min sampling period. ●, Plants inoculated with P. s. pv phaseolicola race 1 (incompatible combination); ○, plants inoculated with virulent race 3 of the pathogen (compatible combination); □, buffer-inoculated plants. The dotted line indicates the maximum level of volatile production seen in the compatible combination. The arrow shows the mean time at which HR cell collapse visible to the naked eye occurred. The data values for the 24-h sampling time were chosen for statistical analysis using a one-way analysis of variance. Mean trans-2-hexenal for HR = 1.9 µg, se = 0.6, n = 5; for susceptible response, 0.2 µg, se = 0.1, n = 5; with buffer, 0.0, se = 0.0, n = 4. Mean cis-3-hexenol in the HR = 10.7 µg, se = 3.3, n = 4; for susceptible response, 0.7 µg, se = 0.3, n = 5; with buffer, 0.1 µg, se = 0.1, n = 4. There were significant differences among the treatments in the amounts of both trans-2-hexenal and cis-3-hexenol ($F_{2,10} = 11.1$ and $F_{2,11} = 8.00$, respectively, both $P < 0.01$). Furthermore, Fisher's tests of the LSDs showed that the significant differences were due to the HR being significantly different from the susceptible response and buffer treatments in both cases. There were no significant differences between the buffer treatment and the susceptible response.

Figure 2. Gas chromatograph of volatiles produced by P. vulgaris cv Red Mexican plants over a 30-min period at 24 h after inoculation. A, HR; B, susceptible response; C, buffer-inoculated control plants.
Antibacterial Activity of Lipid Breakdown Products

Figure 4. MS profiles for substances putatively identified as trans-2-hexenal (A) and cis-3-hexenol (B).

previously seeded with P.s. pv phaseolicola. Control plates (no trans-2-hexenal) showed heavy bacterial growth. Smaller amounts of trans-2-hexenal applied as a saturated buffer solution to filter discs delayed or retarded bacterial growth (Table I). trans-2-Hexenal seemed to exert a bactericidal effect in amounts greater than 16.5 nL. The effect was uniform over the entire plate and not just in the immediate area surrounding the filter disc, showing that the trans-2-hexenal was volatile enough to have a bactericidal effect some distance away from the immediate point of application. When trans-2-hexenal was applied as a buffer-saturated solution solidified in agar plugs, the volatility was reduced and distinct zones of inhibition of bacterial growth could be seen on seeded plates (Fig. 5). Even at the lowest concentration of trans-2-hexenal tested (plug “a” contained 55 nL of trans-2-hexenal) there was clear evidence of bactericidal activity. The trans-2-hexenal appears to have diffused out from the point of application, killing bacteria as it went. The size of the colorless area where bacterial growth was inhibited was dependent on the concentration of bacterial inoculum used to seed the plate. Concentrated inoculum gave rise to a smaller-diameter inhibition and more dilute inocula used to a larger one. This would imply that as the trans-2-hexenal diffused through the agar it was effectively titrated out by the bacteria that it killed. For comparison of results, it is important that the bacteria are fresh, the A600 is known, and that the volume of bacteria added per plate is constant. This plate assay method is superior to the method in which trans-2-hexenal was spotted onto filter discs in that the trans-2-hexenal is localized by being set in agar and gives clear zones of inhibition rather than having a diffuse effect over the entire plate.

When trans-2-hexenal was added to liquid cultures of P.s. pv phaseolicola to concentrations of 8.65 mM, 1.73 mM, and 360 μM, bacterial growth ceased almost immediately in all flasks, and over the next hour the A600 of the cultures dropped, indicating lysis of the bacteria. Over the next 6 h, the control culture with no added trans-2-hexenal grew normally. Loopfuls of culture from each flask were then spread onto NAC plates and incubated as before. After 2 d of incubation at 25°C, some bacteria were shown to have remained viable in those cultures that contained 1.73 and 0.36 mM trans-2-hexenal. At lower concentrations (173–5.8 μM), prepared by adding trans-2-hexenal-saturated buffer to the medium, there appeared to be a direct relationship between the amount of trans-2-hexenal present and the retardation of bacterial growth (Fig. 6).

Table I. The effect of trans-2-hexenal-saturated buffer pipetted onto filter discs on the growth of bacteria on NAC plates

<table>
<thead>
<tr>
<th>nL of trans-2-Hexenal in 50 μL of Buffer Added to Each Plate</th>
<th>Growth after 1 d of Incubation at 25°C</th>
</tr>
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<tbody>
<tr>
<td>0.03</td>
<td>++++</td>
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<tr>
<td>0.33</td>
<td>++++</td>
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<tr>
<td>1.7</td>
<td>+++</td>
</tr>
<tr>
<td>16.5</td>
<td>++</td>
</tr>
<tr>
<td>83.3</td>
<td>+</td>
</tr>
<tr>
<td>167</td>
<td>No growth</td>
</tr>
<tr>
<td>Control*</td>
<td>++++</td>
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</tbody>
</table>

* Buffer only (50 μL).

EM of trans-2-Hexenal-Treated Bacteria

Bacteria exposed to 2.2 mM trans-2-hexenal showed considerable disruption to membranes and cell constituents, and the DNA appeared to have been precipitated (Fig. 7A). From the appearance of the cells and the evidence of a decrease in
Figure 6. Growth of *P. s. pv* phaseolicola in liquid cultures in the presence of trans-2-hexenal. The increase in $A_{600} (OD_{600})$ is shown for cultures treated with various amounts of trans-2-hexenal and the control (no trans-2-hexenal) at selected time points during the incubation period. The scales for trans-2-hexenal concentration and time are not linear.

Following the addition of trans-2-hexenal, we concluded that cell lysis had occurred. The appearance of control bacteria (no trans-2-hexenal treatment) is shown in Figure 7B.

**Effect of cis-3-Hexenol in Agar Plugs on Bacterial Growth**

In comparison with trans-2-hexenal, cis-3-hexenol was much less inhibitory to bacterial growth (Fig. 8). Like trans-2-hexenal, cis-3-hexenol was fairly insoluble in aqueous solutions. However, cis-3-hexenol caused inhibition only when it was added to agar plugs in amounts greater than 1 μL. Thus, although cis-3-hexenol did possess some antibacterial activity, it was approximately 20 times less effective than trans-2-hexenal.

**Effect of Traumatic Acid on Bacterial Growth in Shake Culture**

At the concentrations of traumatic acid used in this experiment (1.88, 0.94, and 0.47 mm), there was no apparent difference in bacterial growth in comparison with the control flask. It was impossible to experiment at higher concentrations because of the insolubility of traumatic acid, but compared with trans-2-hexenal, which is active at low concentrations, traumatic acid seemed to have no effect on growth of *P. s. pv* phaseolicola.

**Bacterial Growth in Vivo**

Figure 9 shows that, under the conditions of the experiment, bacteria in the compatible and incompatible combinations...
tions grow exponentially over the first 24 h after inoculation. In the compatible combination, the bacteria have reached their maximum density of approximately $1 \times 10^7$ cfu cm$^{-2}$ of leaf by 48 h after inoculation, but after 24 h, as the density approaches this level, the rate of multiplication slows. In comparison, in the incompatible combination the growth rate is suppressed even over the first 24 h after inoculation. Thus, the maximum density (approximately $6 \times 10^3$ cfu cm$^{-2}$ of leaf) was reached by 24 h after inoculation, and a slight subsequent decline was then observed.

**DISCUSSION**

We previously demonstrated increases in extractable lipoxygenase activity in bean leaves undergoing an HR induced by *P. syringae* pv *phaseolicola* and also measured increases in ethane evolution (Croft et al., 1990) and pentane evolution from intact leaves (Croft, 1990). Because alkane gas production is a measure of lipid peroxidation (Konze and Elstner, 1978; Kunert and Tappel, 1983; Slater, 1984), we concluded that this was evidence for the occurrence of in vivo lipid peroxidation during the HR. Ethane is produced from linolenic acid (Konze and Elstner, 1978), whereas pentane is produced from linoleic acid (Kunert and Tappel, 1983). Both fatty acids are common constituents of *P. vulgaris* membranes (Epston and Deverall, 1968; Hoppe and Heitefuss, 1974). Increases in ethane and pentane were detected in the HR by 6 to 8 h after inoculation, but for these experiments alkane gas evolution was measured by enclosing detached leaves in small air-tight chambers for 2 to 3 h and then analyzing small aliquots of withdrawn air by GC (Croft, 1990; Croft et al., 1990). In the present work, the analysis of other fatty acid hydroperoxide metabolites was facilitated by the use of a "head-space" procedure with Tenax columns to collect volatiles given off from intact leaves (Jüttner, 1987). This proved an excellent, noninvasive method for measuring the in vivo evolution of the substances of interest. This is particularly important because grinding leaves results in the de novo production of trans-2-hexenal (Matthew and Galliard, 1978), making measurements for comparisons between the different treatments more susceptible to artifacts. Collection of volatiles emitted from inoculated plants and their analysis by GC-MS showed that during the HR of *P. vulgaris* to an avirulent race of *P. syringae* pv *phaseolicola*, various C$_3$ aldehydes and alcohols that are produced on the lipoxygenase pathway from the 13-hydroperoxide of linolenic acid (Vick and Zimmerman, 1984, 1987a, 1987b) were detectable by 15 h after inoculation. Substances identified were cis-3-hexenal, trans-2-hexenal, cis-3-hexenol, and trans-2-hexenol (Fig. 2). Plants inoculated with virulent bacteria produced much lower levels of these substances at 24 h after inoculation, and buffer-inoculated plants hardly produced them in detectable quantities at all (Figs. 2 and 3).

Interestingly, we were unable to detect any trans-2,cis-6-nonadienal in the headspace atmosphere of the inoculated plants. This substance arises by hydroperoxide lyase action on the 9-hydroperoxide of linolenic acid. Autoxidation of linolenic acid produces a mixture of the 9- (approximately 37%), 12- (approximately 9%), 13- (approximately 10%), and 16-hydroperoxides (approximately 45%) (Gunstone, 1967, 1986), whereas lipoxygenase activity in bean leaves yields predominantly the 13-hydroperoxide (Matthew and Galliard, 1978). However, the 9-hydroperoxide is converted by a lyase activity, which is constitutive in bean, to yield, ultimately, trans-2,cis-6-nonadienal (Matthew and Galliard, 1978). Thus, the absence of any detectable trans-2,cis-6-nonadienal (sensitivity of detection down to 8 ng) suggests a high degree of specificity to the mode of lipid peroxidation occurring during the HR. Thus, we conclude that the substances we found were formed from the breakdown of linolenic acid as a result of in vivo lipid peroxidation occurring during the HR, most probably initiated enzymically via lipoxygenase activity, although the contribution of autoxidative processes cannot be ruled out categorically.

Aldehydes, and particularly trans-2-hexenal, are quite reactive and have pronounced effects on biological systems (Schauenstein et al., 1977). In addition, trans-2-hexenal has been reported to be inhibitory to various fungi, insects, and mites (Lyr and Basaniak, 1984) and to protozoa (von Schildknecht and Rauch, 1961). Consequently, it seemed possible that, particularly in light of the extensive production of volatiles in the resistance response, trans-2-hexenal and perhaps other substances on the linolenate cascade were part of the plant's defense against pathogenic bacteria. In liquid cultures, trans-2-hexenal showed a measurable effect on bacteria at approximately 6 µM (Fig. 6). Because of its volatility, it was able to kill bacteria over the whole surface of agar plates when spotted onto small filter discs (Table I). However, when trans-2-hexenal was sealed in agar plugs, its volatility was reduced and distinct zones of bacterial cell death around the agar plugs were visible (Fig. 5). Similarly, agar plate assays showed that cis-3-hexenol possessed antibacterial activity, but it was much less bactericidal than trans-2-hexenal (Fig. 7). Thus, 55 nL of trans-2-hexenal killed bacteria in the agar-plug plate assay, whereas the lowest detectable bactericidal amount for cis-3-hexenol in this assay was 1 µL, i.e. it...
was approximately 20 times less effective than trans-2-hexenal. Cis-3-hexenal, which readily isomerizes to trans-2-hexenal (Hatanaka and Harada, 1973), was also evolved in substantial amounts in the HR (Fig. 2). However, it was not tested for bactericidal activity because it is relatively unstable and not commercially available.

Exactly how trans-2-hexenal exerts its antimicrobial activity is not known. The electron micrographs of trans-2-hexenal-treated bacterial cells (Fig. 7A) appear to show precipitation of DNA into strands and lysis of some cells, which is not seen in the untreated control cells (Fig. 7B). It has been shown that the antiviral activity of saturated aldehydes was negligible when compared with that of the corresponding aldehydes with an αβ-unsaturated group (Schauenstein et al., 1977), and it was concluded that this was the active portion of the molecule. Antiviral activity of trans-2-hexenal was thought to be due to the effect of protein cross-linking and modifications of sulfhydryl and amino groups (Schauenstein et al., 1977). The sulfhydryl groups of proteins and of compounds like GSH are generally essential to their function. Detoxification of αβ-unsaturated aldehydes is possible by enzyme-catalyzed addition to GSH, oxidation to less toxic unsaturated acids, or reduction to alcohols (Schauenstein et al., 1977), although the alcohols, such as cis-3-hexenol, still possess some antimicrobial activity (Fig. 8).

Hydroperoxide lyase activity produces the C₁₂ and C₆ moieties that are further metabolized to traumatic acid and the various C₄ aldehydes and alcohols, respectively (Fig. 1). However, traumatic acid was not detected under the conditions used here for GC-MS. Traumatic acid, which is produced from both linoleic acid and linolenic acids, is associated with wounding and cell proliferation in plants (Vick and Zimmerman, 1987a) and causes abscission in cotton buds (Strong and Kruitwagen, 1967). It appeared to have no effect on the rate of growth of bacterial cells in liquid culture at the concentrations tested, and we conclude that a direct antibacterial role is unlikely. Other effects of traumatic acid on the plants themselves, e.g., the induction of phytoalexins, were not measured.

The actual quantity of volatiles produced in leaves is almost certainly greater than that measured in the head-space procedure, which can only detect those that escape from the plant. Thus, certain amounts will react with cell contents, bacteria, etc. and will in effect be titrated out. A similar situation applies in the agar-plug assay, where an increase in concentrations may be measured. The behavior of the bioassay is an indicator of the rate of production and consumption of volatiles at the plant tissue level.

The data presented here show the earliest time at which we can detect release of volatiles from diseased leaves and do not mean that biologically significant quantities of these active substances are not being produced earlier.

Thus, we hypothesize that the lipid-derived C₄-volatiles produced during HR cell death in plants may play an antipathogen role in plant defense responses, behaving like volatile phytoalexins. The early timing of production of the C₄ aldehydes and alcohols may mean that they are important in the early plant resistance responses because they are detectable in relatively high concentrations at between 15 and 24 h after inoculation. By 15 to 24 h after inoculation with avirulent bacteria, the primary leaves showed extensive hypersensitive cell collapse. At this stage, each pair of primary leaves was evolving approximately 2.2 µg of trans-2-hexenal and 10.3 µg of cis-3-hexenol per 30 min of collection time. This would be equivalent to approximately 40 µg of trans-2-hexenal and approximately 185 µg of cis-3-hexenol, respectively, over the 9-h period. With such large amounts of the volatile substances being lost from leaves, it seems fair to assume that the effective concentrations, particularly for trans-2-hexenal, at sources within the leaves might be in the range that would affect bacterial growth. Duniway (1973) showed that P. syringae induced stomatal closure in tobacco during an HR. Assuming this is also true in bean, it would further support the suggestion that toxic levels of volatiles might be reached in the intercellular spaces.

Earlier work with this pathosystem showed that numbers of both virulent and avirulent bacterial isolates increased in leaves for the first 24 h after inoculation and that around the time of HR cell collapse the rate of multiplication of the avirulent isolate began to decline (Lyon and Wood, 1975, 1976). Similar trends were shown for the growth of compatible P. syringae and the nonhost plant pathogenic bacterium P. coronafaciens (HR-eliciting) in bean tissues (Daub and Hagedorn, 1980). Because the above studies employed different inoculation procedures, inoculum concentrations, and plant growth conditions, we carried out our own studies on the growth of virulent and avirulent isolates of P. syringae phaseolicola in bean leaves.

In agreement with the earlier studies (Lyon and Wood, 1975, 1976; Daub and Hagedorn, 1980), our results also showed a divergence in growth rates between virulent and avirulent races over the first 48 h after inoculation. Growth of the avirulent race 1 cells appeared to be depressed quite early compared with virulent race 3 cells, and race 1 cells had reached their maximum leaf density by 24 h after inoculation (Fig. 9). In the compatible combination the bacteria reached the maximum density usually observed for this pathosystem of approximately 1 x 10⁸ cfu cm⁻² of leaf (Young, 1973) by 48 h after inoculation, but after 24 h, as the density approaches this level, the rate of multiplication slows (Fig. 9). Thus, it seems possible that trans-2-hexenal and perhaps to a lesser extent the other lipid-derived volatiles may be in part responsible for the early suppression of bacterial multiplication observed in the HR, either by killing a proportion of the bacteria directly or by suppressing the growth rate of the population as a whole. However, to be an effective component of the plant's defense repertoire. However, the well-described isoflavonoid phytoalexins of bean (e.g., phaseollin, coumes-
important in resisting colonization of necrotic tissues by secondary, opportunistic invaders. Because trans-2-hexenal damaged the plants and at high concentrations caused necrosis, it is of course possible that it contributes to causing the host cell death seen in the HR. However, large amounts were produced only after visible necrosis had occurred, and it seems likely that trans-2-hexenal is produced rather as a consequence of cell collapse. It does not appear to play a role in signal transduction because hexenal-treated plants did not accumulate phytoalexins.

It is interesting that no saturated C6 alcohols or aldehydes, which would derive from linoleic acid-13-hydroperoxide, were detected. Because inoculated plants evolved pentane (Croft, 1990), peroxidation of linoleic acid does occur during the HR; therefore, we conclude that in the HR any linoleic acid 13-hydroperoxide that forms must be metabolized via other pathways, perhaps those utilizing dehydrase (isomerase and cyclase) activities. Thus, the metabolites would not feed into the pathway leading to these C6 compounds. This observation, together with the apparent lack of production of trans-2-cis-6-nonadienal (see above), suggests that the peroxidation of fatty acids, which specifically accompanies the HR in bean, is under tightly regulated metabolic control.

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