Hexanal, *trans*-2-Hexenal, and *trans*-2-Nonenal Inhibit Soybean, *Glycine max*, Seed Germination

Harold W. Gardner,* David L. Dornbos, Jr.,‡ and Anne E. Desjardins

Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604

Hexanal, which is biosynthesized in plants by the action of lipoxygenase and hydroperoxide lyase on linoleic acid, inhibited the germination and subsequent growth of soybean, *Glycine max* cv. Century. Hexanal, vaporized in air with a flow-through system (100 mL/min), was continuously delivered to germinating seeds. Only 0.9 μg of hexanal/mL of air was required to inhibit seed germination by 50%; nearly 100% inhibition occurred with a dose of 1.8 μg of hexanal/mL of air. In the absence of hexanal, fungal growth was usually present on the testa, but at sublethal doses of hexanal, the seedlings, including the residual testa, were largely devoid of fungal growth. The relative toxicities of hexanal and two other aldehydes formed as a result of lipoxygenase–hydroperoxide lyase action, *trans*-2-hexenal and *trans*-2-nonenal, were compared by a short time exposure in a Petri dish bioassay. The order of inhibition of growth was *trans*-2-hexenal > hexanal > *trans*-2-nonenal when germinating seeds were exposed to saturating vapors of the aldehydes. However, when the relative vapor pressure of the aldehydes was accounted for, the order of toxicity was calculated to be approximately *trans*-2-nonenal ≥ *trans*-2-hexenal > hexanal.

INTRODUCTION

Tissue damage to imbibed soybean seeds or mature plants triggers the lipoxygenase–hydroperoxide lyase enzymic pathway converting linoleic and linolenic acids into hexanal and *cis*-3-hexenal, respectively (Hatanaka et al., 1978; Matoba et al., 1985; Sekiya et al., 1986). The *cis*-3-hexenal is usually isomerized to *trans*-2-hexenal both enzymically and nonenzymically (Gardner, 1989). These aldehydes are responsible for the green/beany odor that develops in damaged soybeans. Several investigators have shown that hexanal and *trans*-2-hexenal are toxic to microorganisms, particularly fungal species (Major et al., 1960; Schildknecht and Rauch, 1961; Nandi and Fries, 1976; Gueldner et al., 1985; Urbasch, 1987). However, these volatile aldehydes also are toxic to higher plants, which themselves are hosts to the aldehyde biogenesis. For example, hexanal inhibited the germination of several seed species (Nandi and Fries, 1976; French and Leather, 1979; Bradow and Connick, 1988).

Prior studies have not fully defined the concentration of hexanal required to inhibit the germination of seeds, nor have they described the toxicity of hexanal to soybeans. The current study delineates the dose–response inhibition of soybean seed germination and growth when hexanal is delivered as a gas vaporized in air by a flow-through apparatus. In addition, the relative toxicity of two other products originating from lipoxygenase–hydroperoxide lyase action, *trans*-2-hexenal and *trans*-2-nonenal, is described.

MATERIALS AND METHODS

Continuous Flow Hexanal. Germinating soybeans, *Glycine max* cv. Century, were exposed to various concentrations of hexanal (99% from Aldrich Chemical Co., Milwaukee, WI) vaporized in air for 3 days in the dark at 25 °C. Soybean seeds, produced in 1987 and certified by the Illinois Crop Improvement Association, were obtained locally. As shown in Figure 1, humidified air was mixed with a second stream of humidified air that was saturated with hexanal vapor. Since Tygon tubing absorbed hexanal vapors, the hexanal vaporizing chambers and all apparatus downstream were glass or Teflon. A few nonstandard glass fittings were connected with Tygon tubing sleeves, but only with the Teflon tubing fitted flush to the connecting glass tubes. Headspace gas was sampled through Mininert Teflon valves obtained from Pierce Chemical Co., Rockford, IL. The gas mixture was delivered at a final flow rate of 100 mL/min to a 2.9-L enclosed chamber that contained 50 soybeans, 25 on each of two platforms covered with moistened multilayered absorbent paper (Kimpac, Kimberly-Clark Corp.). The chamber was humidified by a 100-mL H_2O reservoir below the seed platforms. Flow rates of the two gas streams were maintained by Model 8744 flow controllers (Brooks Instrument, Hatfield, PA). Except in experiments designed to test the effect of hexanal on indigenous populations of microorganisms present on the seeds, the seeds were surface sterilized by soaking for 10 min with 10% Chlorox prior to incubation of the seeds to reduce the growth of microorganisms. Because hexanal rapidly autoxidized over the 3-day germination period, 0.05% 2,6-di-*tert*-butyl-*p*-cresol (BHT) from Baker Chemical Co., 1 mM β-carotene (Sigma Chemical Co.), and 0.012% ethylenediaminetetraacetic acid (EDTA) were added to the hexanal in the vaporizing chamber to maintain a stable level of hexanal vapor. Unused hexanal was discarded after each experiment. The concentration of hexanal in the enclosed chamber was quantified each day during the 3-day germination period by gas–liquid chromatography (GLC) of headspace samples.

After treatment, the number of germinated seedlings (protrusion of the radicle through the seed coat) was counted. Subsequently, the seedling axes (hypocotyl plus epicotyl) were dried at 70 °C for 24 h and weighed.

Microbial Techniques. To assess microbial growth indigenous to seeds, one germinated seed was transferred to each of six wells of multiwell tissue culture plates (Becton-Dickinson Co., Lincoln Park, NJ) by asceptic technique. Each culture well (3.5 × 1.7 cm) contained 2 mL of 5% agar in water. The culture plates were placed in flow-through chambers (Figure 1) supplied either with hexanal-amended air or air only. Micro-

* To whom correspondence should be addressed.
‡ Present address: Ciba-Geigy Seed Division, P.O. Box 18300, Greensboro, NC 27419-8300.

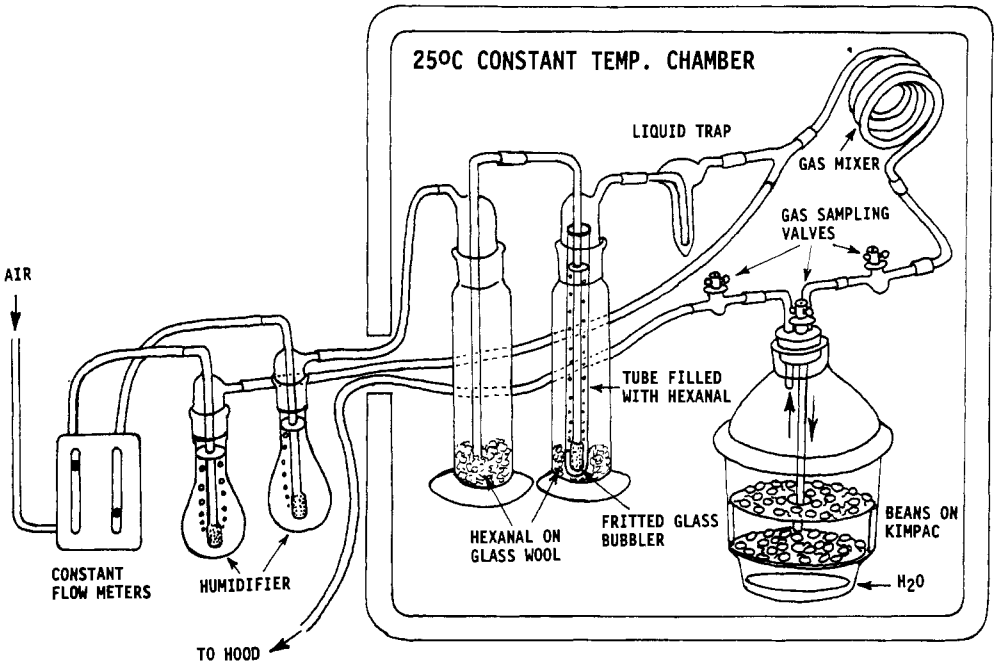

Figure 1. Flow-through apparatus supplying various concentrations of hexanal vapor in air to germinating soybeans.

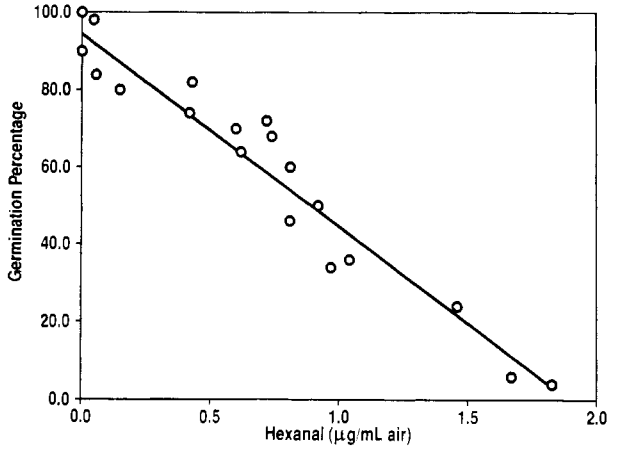

Figure 2. Effect of hexanal concentration in the vapor phase on the germination of soybean seeds.

bial growth was examined by microscopy, and fungi were identified by spore morphology.

Comparative Aldehyde Toxicity. Toxicities of hexanal, *trans*-2-hexenal (99%, Aldrich), and *trans*-2-nonenal (97%, Aldrich) to soybean seed germination were compared in a Petri dish bioassay. Soybean seeds were exposed to saturating levels of aldehyde vapors for various lengths of time, removed from the vapors, and then germinated for additional time in the absence of the aldehydes. Prior to aldehyde exposure, the seeds were either not imbibed or imbibed at room temperature for either 6 or 12 h. Ten seeds were placed on moistened Kimpac in replicated 9 cm × 1.5 cm Petri plates. The seeds were then exposed to aldehyde vapors (hexanal, *trans*-2-hexenal, or *trans*-2-nonenal) from a center well containing an excess of the aldehyde on a wad of cotton in sufficient quantity to saturate the air space (50 μL). The Petri plates were sealed in plastic bags for the prescribed exposure period. After exposure, the seeds were removed from the Petri plates, rinsed with H_2O, and transferred to aldehyde-free plates containing moistened Kimpac for a 3-day germination at 26 ● 1 °C. The 3-day total germination included aldehyde exposure time plus postexposure germination, but not preexposure imbibition time. Seeds with protruded radicles were counted as germinated.

GLC. Headspace samples were collected with a 1-mL gastight syringe (Dynatech Precision Sampling Corp., Baton Rouge, LA), and the collected gas vapors were separated by GLC (Spectra-Physics Model SP-7100 gas chromatograph) using an open tubular capillary column (25 m × 0.25 mm) coated with a 0.25-μm film (007 CPS-2) from J & S Scientific (Crystal Lake, IL). The carrier gas flow was a 100 to 1 split at 100 mL/min (1 mL/min actual flow); the oven temperature increased from 45 to 55 °C at 5 °C/min. Hexanal eluted after 1.7 min. The peak area of hexanal was quantified by comparison with the response of injected samples of standard hexanal dissolved in hexane. With mixtures of hexanal, *trans*-2-hexenal, and *trans*-2-nonenal, the temperature program was 45 to 55 °C at 5 °C/min, followed by 15 °C/min to 105 °C, and held at 105 °C for 2 min. Elution was hexanal, *trans*-2-hexenal, and *trans*-2-nonenal at 1.7, 2.7, and 5.9 min, respectively.

To determine the relative vapor pressure of hexanal, *trans*-2-hexenal, and *trans*-2-nonenal, an equimolar mixture of the three was mixed and a 4-μL sample of the mixture was transferred to a 50-mL gastight vessel for incubation at 26 °C. GLC of headspace samples from the vessel afforded relative peak areas that were adjusted in terms of response factors by injection of the equimolar mixture dissolved in ethyl ether.

RESULTS

Effect of Continuous Flow Hexanal. A variety of hexanal concentrations was delivered to germinating soybean seeds for 3 days with the continuous flow device shown in Figure 1. GLC of headspace samples permitted accurate monitoring of the actual hexanal content. Exposure to increasing concentrations of hexanal vapors reduced the percentage germination of the seeds (Figure 2); a concentration of 0.9 μg of hexanal/mL of air was required to reduce germination by about 50%. At 25 °C a 1.05 mM solution of hexanal suspended in water by 0.01% Tween 20 equilibrates with headspace at 0.9 μg of hexanal/mL of air (2:1 headspace to solution ratio), which gives an estimate of the aqueous hexanal concentration required to inhibit germination at this level. No germination occurred above about 1.8 μg of hexanal/mL of air (Figure 2).

There was a notable inhibition of growth in the seedlings that had germinated in the presence of hexanal as measured by the dry weight of seedling axes (Figure 3). These data were plotted either on a weight per test seed basis, including ungerminated seeds (lower plot), or on a weight per germinated seed basis only (upper plot). The latter plot indicates the actual growth inhibition per germinated seedling, whereas the former plot reflects the combined effects of germination and growth inhibition.

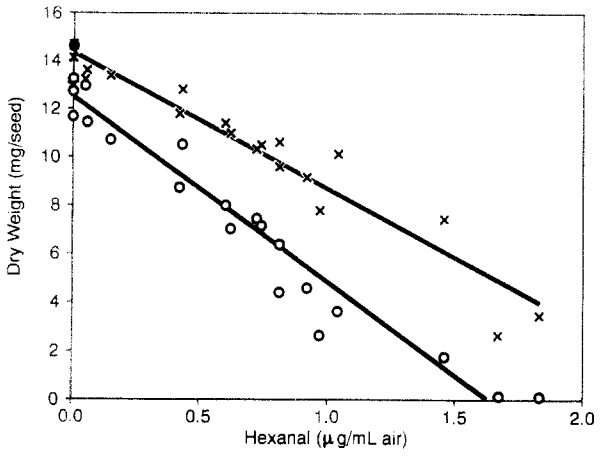

Figure 3. Effect of vapor-phase hexanal concentration on the axis dry weight of germinated soybean seeds with radicle protrusions plotted as milligrams per test seed, including ungerminated seeds (O), and milligrams per germinated seed only (×).

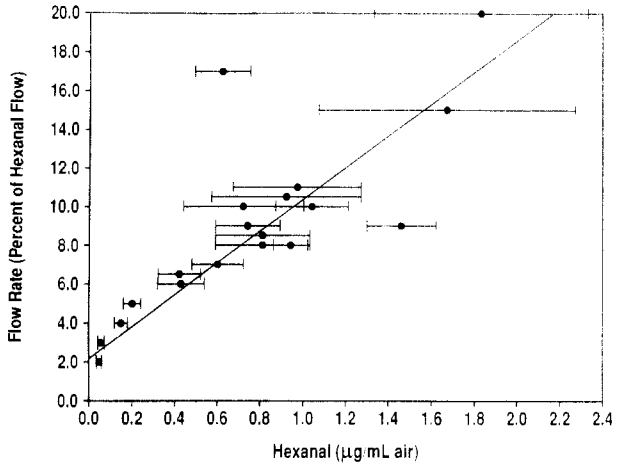

Figure 4. Effect of percent hexanal flow rate (total of 100 mL/min) on hexanal delivery to soybean germination chamber. Linear regression of hexanal includes all data points, including two points not shown (flow rates of 25% and 50% hexanal makeup with hexanal contents of 4.50 ± 0.52 and 5.49 ± 1.02 μg/mL of air, respectively); $R^2 = 0.86$.

In addition to lack of growth, the seedlings exposed to hexanal occasionally developed a curled and/or thickened hypocotyl/root, especially at higher doses.

Although the flow-through apparatus performed the desired task, the system was not without its eccentricities. Initial experiments using hexanal directly from the supplier in the apparatus shown in Figure 1 gave decreasing levels of hexanal over the 3-day time required to germinate the soybeans, indicating that hexanal was autoxidizing. When an antioxidant mixture of β-carotene, BHT, and EDTA was used to prevent autoxidation, hexanal persisted for the length of the experiment.

Occasionally, the actual content of hexanal in the germination chamber was not fully predictable. The amount of hexanal supplied could be generally controlled by the percent makeup of hexanal-saturated air with air only at a total flow rate of 100 mL/min (Figure 4). As seen by a few outlier points in Figure 4, it was concluded that continuous analyses of hexanal concentration in the headspace from the chamber were absolutely essential to the collection of reliable dose–response data. Also, the coefficient of variation was inordinately large (Figure 4). Because the amount of hexanal in micrograms per milliliter of air was reported as the mean and standard deviation of all headspace samples taken over the time of

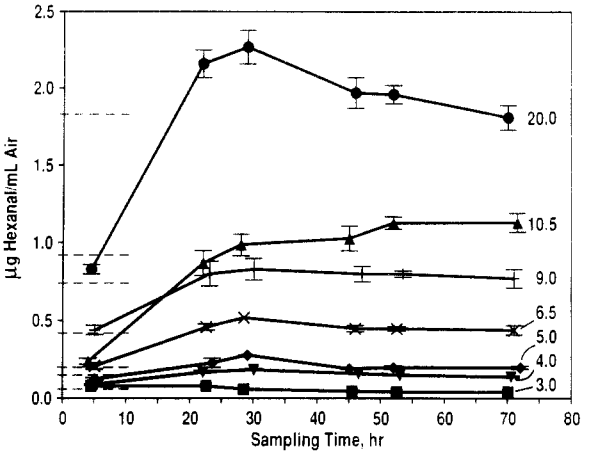

Figure 5. GLC analyses of hexanal content in the headspace of the germination chamber as a function of time using various rates of hexanal makeup. Flow rates in percent hexanal makeup are given by the values on the right. The dashed lines on the left indicate the mean value of the hexanal analyses for a given flow rate.

germination from the germination chamber, this large variance can be largely ascribed to low levels of hexanal found in the earliest headspace samples (Figure 5). In theory, three complete exchanges of atmosphere should bring the seed chamber up to 95% of the maximum hexanal content. For the 2.9-L gas space in the incubation chamber, a 95% level should have been achieved after only 87 min at the flow rate of 100 mL/min. This level was not attained; thus, it can be surmised that the reservoir of water on the bottom of the chamber and in the Kimpac, as well as the Kimpac itself, may have required additional time to equilibrate with the gas phase. This was corroborated by the fact that headspace samples from the inlet consistently had greater hexanal concentrations than samples from the germination chamber.

Effect of Hexanal on Indigenous Microbes. Under all but the lowest hexanal levels, the seeds or seedlings appeared to be free of overt fungal infestation after 3 days of germination. Without hexanal, fungi grew primarily on the testa of the seeds or seedlings. To test this phenomenon in more detail, seeds (untreated with Chlorox) were germinated for 3 days as usual with and without hexanal. The flow rate of the hexanal treatment was set at 100 mL/min with 9.5% hexanal-saturated air makeup. The with-hexanal treatment resulted in 92% germination of seeds with no fungal growth by visual inspection. In the absence of hexanal, germination was 100%, and fungal growth was evident. In particular, the residual testa of one seedling was completely covered with sporulating *Alternaria*, and the testa of other seeds or seedlings were sparsely covered with fungal hyphae and/or spores. Subsequently, individual seedlings from each treatment were randomly transferred to water–agar culture plates for a further 5-day incubation with or with hexanal (total of four treatments). As seen in Table I, bacteria were present in all cases, but fungal growth was highly dependent on the treatment. The initial 3-day dose of hexanal appeared to be the most effective in inhibiting fungal growth, whereas a subsequent 5-day incubation with hexanal was ineffective, apparently because fungal growth was already initiated by a prior no-hexanal treatment. Under this latter set of conditions, only sporulation was somewhat inhibited.

Comparative Aldehyde Toxicity. Because of the expense of testing other hydroperoxide lyase products, *trans*-2-hexenal and *trans*-2-nonenal were not utilized in

Table I. Occurrence of Microorganisms on Soybean Seedlings[a] with and without Hexanal Treatment

hexanal[b]		bacteria	fungi: hyphae	fungi: spores	fungi: identification
+	+[c]	12/12[d]	0/12		
+	−[c]	12/12	6/12 (sparse)	1/12 (sparse)	*Penicillium* or *Aspergillus*
−	+	12/12	11/12 (heavy)	8/12 (sparse)	*Fusarium, Alternaria, Penicillium, Aspergillus*, and others
−	−	12/12	12/12 (heavy)	12/12 (heavy)	*Fusarium, Alternaria, Penicillium, Aspergillus*, and others

[a] Soybeans were not pretreated with 10% Chlorox to reduce microorganisms prior to germination. [b] The first + or − symbol indicates that soybeans were treated with or without hexanal for 3 days. Subsequently, the seedlings were transferred to water–agar culture plates for 5 additional days of incubation with or without hexanal, which is denoted by the second + or − symbol. Hexanal treatment was 9.5% hexanal-saturated air makeup (total air flow of 100 mL/min) that was analyzed to contain 0.94 ± 0.33 μg hexanal/mL of air; the no-hexanal treatment was 100 mL/min air only. [c] One seed of 12 did not germinate. [d] Number of occurrences per 12 seeds or seedlings.

Table II. Increased Toxicity of Hexanal after Imbibition

time of postimbibition exposure to hexanal, h	seedling dry weight,[a] mg/seed, at imbibition time, h: 0	6	12	% germination at imbibition time, h: 0	6	12
0	7.8 ± 2.8	8.5 ± 1.2	10.8 ± 1.2	67	83	80
0.25	11.9 ± 1.5	10.5 ± 2.3	10.4 ± 1.8	83	80	90
0.5	10.1 ± 2.5	9.2 ± 1.4	10.2 ± 0.7	87	83	90
0.75	10.9 ± 1.7	6.3 ± 2.0	8.5 ± 3.5	83	67	73
1	9.0 ± 1.0	7.2 ± 1.1	4.9 ± 1.7	70	63	60
1.5	9.5 ± 6.2	5.2 ± 2.9	4.6 ± 2.0	63	57	63
2	8.3 ± 3.7	6.4 ± 0.5	3.2 ± 0.7	67	75	53
4	7.9 ± 1.4	2.9 ± 0.8	2.4 ± 1.0	67	40	40
6	10.6 ± 1.9	1.6 ± 0.6	1.9 ± 1.3	80	23	33

[a] n = three measurements of 10 seedlings each; dry weight of seedling minus cotyledons. Seeds were germinated for 3 days at 26 ± 1 °C. Only the axes of germinated seeds with radicle protrusions were included in the dry weight; however, the dry weight was calculated in terms of milligrams per test seed, including ungerminated seeds.

the flow-through apparatus. For this reason, an alternative method was developed to compare the toxicity of hexanal vapors with vapors of *trans*-2-hexenal and *trans*-2-nonenal. Thus, soybeans were exposed to either hexanal, *trans*-2-hexenal, or *trans*-2-nonenal vapors in Petri plates on moistened Kimpac for various times up to 6 h, removed from the vapors, and germinated in an aldehyde-free environment to compare the effect on seedlings.

Hexanal had little effect on soybean seeds unless they received a pre-imbibition treatment (Table II). Subsequent experiments were completed with soybean seeds that were pre-imbibed for 12 h.

Pre-imbibed seeds were exposed to vapors of hexanal, *trans*-2-hexenal, and *trans*-2-nonenal in the Petri plate assay. Seed germination and seedling growth were reduced most by *trans*-2-hexenal, followed by hexanal, and *trans*-2-nonenal. This order of toxicity is largely affected by the relative vapor pressure of the respective aldehydes. By GLC of headspace vapors from an equimolar mixture of hexanal, *trans*-2-hexenal, and *trans*-2-nonenal, the resultant peak areas were adjusted to account for response factors obtained by GLC of an ether solution of the same equimolar mixture of the aldehydes. The relative vapor pressures obtained in this way were adjusted to hexanal set at unity: hexanal, 1; *trans*-2-hexenal, 0.72; *trans*-2-nonenal, 0.034. According to the gas law, relative vapor pressures are equivalent to molar ratios. By use of these values, a better assessment of molar toxicities can be obtained. From the data in Figure 6, linear regression of exposure time against seedling dry weight for the three aldehydes permitted an estimation of the time of exposure required to reduce seedling dry weight by half as follows: hexanal, 2.3 h; *trans*-2-hexenal, 0.46 h; *trans*-2-nonenal, 6.6 h (the longest exposure time for *trans*-2-hexenal, 1.5 h, and *trans*-2-nonenal, 21 h, was omitted from the regression as falling beyond the expected killing time). Although one could question the efficacy of applying molar dosages to modify exposure time values, it is evident that *trans*-2-nonenal is much more toxic than Figure 6 suggests. It is more reasonable to propose that the molar toxicity is as follows: *trans*-2-nonenal ≥ *trans*-2-hexenal > hexanal.

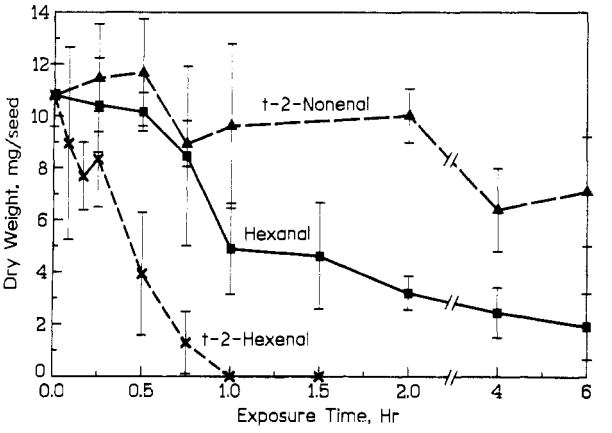

Figure 6. Effect of exposure time to vapors of either hexanal, *trans*-2-hexenal, or *trans*-2-nonenal on seedling axis dry weight (milligrams per test seed, including ungerminated seeds) of soybeans that were pre-imbibed 12 h before exposure. Not shown are *trans*-2-nonenal exposure times of 12 and 21 h yielding seedling axis dry weights of 1.8 ± 1.1 and 0.14 ± 0.24 mg, respectively. Linear regression of exposure time against mean dry weight values furnished R^2 = 0.68, 0.97, and 0.91 for hexanal, *trans*-2-hexenal, and *trans*-2-nonenal, respectively (the longest exposure time for *trans*-2-hexenal and *trans*-2-nonenal was omitted in the regression as falling beyond the expected killing time).

DISCUSSION

If one monitors hexanal levels by GLC, the flow-through apparatus shown in Figure 1 is a reliable method of providing seedlings with a known amount of hexanal vaporized in air. Since at least a 6-h imbibition is necessary before treatment with hexanal to demonstrate significant inhibition of soybean seed germination (Table II), the low levels of hexanal in the early stage of germination were probably unimportant. Linear regression of mean hexanal content against flow rate gave a coefficient of determination (R^2) of 0.86 (Figure 4).

Previous studies of hexanal toxicity consisted of static systems where quantities of hexanal, or other aldehydes, were simply added to a desiccator that contained germinating seeds (French and Leather, 1979; Bradow and Connick, 1988). Because the degree of hexanal autoxidation and quantity of vaporized hexanal were not determined during the course of the experiment, this static method must be considered semiquantitative. Nandi and Fries (1976) directly applied 14 different aldehydes, including hexanal, to seeds and then removed the seeds from the aldehydes for germination assays. The latter workers found that the aldehydes decreased seed germination, particularly when the seed moisture was increased. Similarly, the present results showed a requirement for imbibition moisture to fully inhibit seed germination in the presence of hexanal.

In determining relative toxicities of hexanal, *trans*-2-hexenal, and *trans*-2-nonenal, it is first recognized that the toxicity of hexanal is known from the flow-through device discussed above. By determining the exposure time required to reduce germination and growth of soybeans by the three aldehydes, we established the relative toxicity order of the aldehydes dependent upon their differences in vapor pressure. A determination of the relative partial pressure of the aldehydes led to the following estimation of relative molar toxicity: *trans*-2-nonenal $\geq$ *trans*-2-hexenal > hexanal.

Inasmuch as the aldehydes in question are produced in plants by the lipoxygenase–hydroperoxide lyase pathway (Gardner, 1989), it is of interest to understand the role these compounds play in the physiological functioning of the plant. It is known that aldehydes are reactive with various biochemicals (Gardner, 1979), which may explain part of their toxic effect on seed germination and seedling growth. However, this would not seem to impart much advantage to the plant. The potential role of these aldehydes as antibacterial or antifungal agents has been postulated previously (Major et al., 1960; Schildknect and Rauch, 1961; Nandi and Fries, 1976; Gueldner et al., 1985; Urbasch, 1987). The previous studies do not offer much information concerning the actual amounts of aldehydes produced by plants versus the effectiveness of these levels in inhibiting the growth of microorganisms. Crushing, macerating, or homogenizing soybean seedlings released negligible but measurable amounts of hexanal (Gardner, unpublished data). Undamaged seedlings also released relatively trace amounts of hexanal. However, 0.4 mM hexanal can be produced in a seedling homogenate provided that 1 mM of the product of soybean lipoxygenase action on linoleic acid, the 13(*S*)-hydroperoxide, is added. In a container with a solution to headspace ratio of 1:2, a 0.4 mM hexanal solution equilibrates with headspace at 0.34 μg/mL of air, which is probably sufficient to inhibit both seedling and fungal growth.

If soybean seedlings use this biochemical pathway to protect themselves against fungal growth, how can sufficient substrate be provided to be effective? Recent research indicated that the rate-limiting step in hexanal biogenesis in seed/seedling homogenates is lipolysis of glycerides to free fatty acids, including linoleic acid, and not the subsequent events of lipoxygenase and hydroperoxide lyase action (Gardner, unpublished data). It is well-known that many species of fungi are efficient in the biosynthesis of lipases (Christensen and Kaufmann, 1969), which they produce to consume the glyceride-rich substrates they parasitize. One can visualize a microenvironment where the penetration of fungal hyphae causes the release of free fatty acids and, subsequently, a localized concentration of hexanal. Thus, a fatal synergism could be involved, much like the induction of plant phytoalexin biosynthesis triggered by the presence of microorganisms. One caveat of the preceding hypothesis is the apparent ineffectiveness of hexanal in inhibiting the growth of fungi at later stages of development (Table I). Differential sensitivity to toxins by various stages of fungal development is not unusual. For example, the phytoalexin medicarpin is fungitoxic to spores and germ tubes, but not mycelium (Smith, 1982).

ACKNOWLEDGMENT

We thank Jan Parsons for completion of most of the technical part and Dr. Gerald R. Leather for a thorough review of the manuscript.

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Received for review November 17, 1989. Accepted February 26, 1990.

Registry No. Hexanal, 66-25-1; *trans*-2-hexenal, 6728-26-3; *trans*-2-nonenal, 18829-56-6.