Evidence Implicating the Lipoxygenase Pathway in Providing Resistance to Soybeans Against *Aspergillus flavus*

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ABSTRACT

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The fungus, *Aspergillus flavus*, secretes lipases while parasitizing oilseed hosts and may contaminate the seed with aflatoxin. We found that exogenous lipase applied to soybean cotyledons results in the generation of volatile aldehydes by the lipoxygenase pathway that halts the growth of fungi. Volatiles generated from untreated homogenized soybeans did not affect the germination of *A. flavus* spores in a closed petri plate assay in which spores were streaked on corn-meal agar and soybean homogenate was placed on the lid of the inverted plate. When lipase (500 units/ml) was added to the soybean homogenate, all fungal spore germination was inhibited. However, the addition of nordihydroguaiaretic acid (a lipoxygenase inhibitor) to the soybean homogenate/lipase mixture partially reversed the inhibition of spore germination. The extent of spore germination was dependent on both the number of soybean cotyledons in the homogenate and the activity of the added lipase. Addition of linoleic or linolenic acids to the homogenates also inhibited spore germination, whereas palmitic, stearic, or oleic acid had no effect, compared to untreated homogenates. Headspace analysis indicated that hexanal, a product of the lipoxygenase pathway with known antifungal activity, was the major volatile generated from lipase-treated homogenates. Soybeans are exceptional among oil-rich seed crops in that they are resistant to *A. flavus* infection and aflatoxin contamination. Our evidence strongly suggests that the lipoxygenase pathway may contribute to this resistance.

The fungus *Aspergillus flavus* Link:Fr. attacks a variety of oil-rich seeds grown in warm, temperate to tropical climates, especially peanut, cotton, and corn and can contaminate the seed with aflatoxin (1). Other commodities that become contaminated with aflatoxin include almonds, Brazil nuts, pistachios, and melon, pumpkin, and sunflower seeds (21), all of which are rich in oil. Remarkably, soybeans, which are also rich in oil, appear to be resistant to *A. flavus* invasion for unexplained reasons (13). We have been investigating the possible role of the lipoxygenase pathway in conferring resistance to fungal invasion in soybeans. Lipoxygenase catalyzes the peroxidation of fatty acids that contain *cis*, *cis*-1,4-pentadiene moieties (5). Hydroperoxide lyases, also

present in soybeans, produce the volatile C_6-aldehydes, hexanal or *cis*-3-hexenal, from the 13-hydroperoxides of linoleic and linolenic acids, respectively (5,8,15). The volatile aldehyde *cis*-3-hexenal is susceptible to isomerization into *trans*-2-hexenal (4,8). Numerous studies have shown that these volatile aldehydes have antifungal and antibacterial properties (3,6,10,11,23). Crushed leaves of nearly every plant will generate *cis*-3-hexenal, evidenced by a grassy smell that is characteristic of *cis*-3-hexenal (12). These volatiles from crushed leaves inhibit growth of *A. flavus* (22) and are generated by infected leaves as a result of the hypersensitive response (3).

Although soybean seeds contain high lipoxygenase and hydroperoxide lyase activities, crushed soybean seeds produce only small amounts of hexanal, presumably because nearly all the fatty acids in soybeans are in triglycerides (16), which do not serve as precursors to volatile aldehydes (8). If either linoleic or linolenic acid is provided to soybean homogenates, abundant volatile aldehydes are generated. Because soybean lipoxygenases are localized subcellularly in the cytoplasm, remote from the lipid storage bodies (20), the function of these proteins, other than serving as seed-storage proteins, has remained unknown. Recently, Gardner et al (6) reasoned that lipase secreted by an invading fungus might be the triggering factor for generation of volatile aldehydes by the lipoxygenase pathway from soybean. Secretion of extracellular lipases and other enzymes by an invading fungus would lyse the soybean cotyledon cell and hydrolyze triglycerides to fatty acids. The release of fatty acids would provide the substrate for lipoxygenase and result in the generation of hexanal, among other products. Such a reaction could inhibit further fungal growth and, thereby, stop the fungal invasion. It is well known that *A. flavus* secretes enzymes with abundant lipase activity when invading oil-seed hosts (19). In this investigation, we tested the hypothesis that the volatile products of the lipoxygenase pathway protect soybeans from invasion by *A. flavus* and that the generation of these volatiles is triggered by the secretion of lipase by the invading fungus.

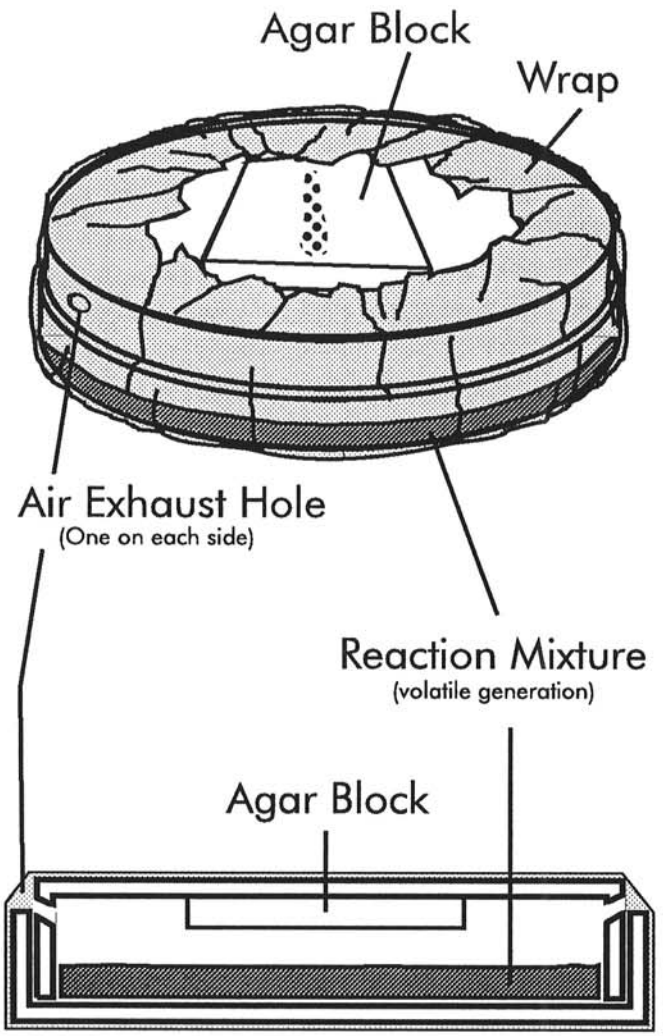

Fig. 1. Inverted petri-plate chamber showing reactive mixture, inoculated agar block, holes for air escape during fitting, and Parafilm wrap.

MATERIALS AND METHODS

Soybeans (*Glycine max* (L.) Merr. 'Williams 82') were obtained from Kelly Seed Co., Peoria, IL. *A. flavus* strain NRRL 6536, an aflatoxin-producing strain, was used for all inoculations and is available from the Agricultural Research Culture Collection, USDA/ARS NCAUR, Peoria, IL. Corn germ (*Zea mays* L.) was isolated from F_2 hybrid seed of Pioneer 3379 grown in Havana, IL, during 1992.

Lipase was purchased from Sigma Chemical Co. (St. Louis, MO; L-4384) and was derived from *Rhizopus arrhizus*. Fatty acids were purchased from Nu Chek Prep, Inc. (Elysian, MN). All other chemicals were obtained from Sigma.

To test the effects of volatiles from soybean homogenates on *A. flavus* spore germination, a closed petri plate-assay system was used (Fig. 1). Soybeans were first soaked for 6 h in tap water at room temperature. Immediately prior to homogenization, soybean testa were removed by dissection, and cotyledons were separated and placed in distilled water on ice. A 0.1-ml droplet of *A. flavus* spores suspended in sterile distilled water (5×10^6 spores per milliliter) was streaked in a straight line (4 cm) onto corn-meal agar in sterile plastic petri plates. Two holes (2 mm diameter) was bored into each side of the bottom section of the plate. Typically, 16 cotyledons (approximately 2.2 g fresh weight, 1.1 g dry weight) were added to 10 ml of 150 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.5, with KOH, along with lipase or fatty acids and homogenized with a Brinkman (Westbury, NY) tissue homogenizer. Petri plates with the fungal spore streaks were inverted, and the bottoms were removed. The soybean homogenates were poured into the inverted lids of the plates and spread to cover the entire surface. The bottoms of the plates were returned to the inverted lids and allowed to settle through the layer of liquid. Excess air inside the plates escaped through the holes bored into the side of the bottom sections. Plates were sealed with Parafilm and placed in an incubator at 30 C. At no point did the soybean homogenate come into direct contact with the fungal spores or their media. After incubation for 16 h, plates were opened, and spore germination was evaluated. Spores were scored as ungerminated, swollen, or germinated. Germ tubes had to be more than 10 μm in length to be scored as germinated. In experiments to test the effect of volatiles from corn-germ homogenates, 32 corn germs were dissected from kernels that had soaked for 6 h. Germs were homogenized with lipase as described for soybeans. Activity of added lipase was varied by dilution of a stock solution.

The effect of the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA; 14) on the generation of volatiles from soybean homogenates containing exogenous lipase affecting *A. flavus* spore germination was tested by adding 0.1 ml of 100 mM NDGA in ethanol to 10 ml of homogenization buffer with four soybean cotyledons (final NDGA concentration of 1 mM). The cotyledons were homogenized, and the homogenate containing the inhibitor was allowed to sit for 5 min before lipase (1,000 units/ml) was added. Homogenates were poured into plate lids and sealed in the presence of spores as indicated previously.

For headspace analysis, soybean homogenates were prepared as described above, except that after homogenization 2-ml aliquots were removed, placed in 6 ml of Reacti-Vials (Pierce Chemical, Rockford, IL) with Teflon gas-tight stopcock valves, and put into a water bath. At timed intervals, vials were sampled for headspace gases with a 2-ml gas-tight syringe. Gases (2 ml) were injected into a gas chromatograph, and volatiles were detected by a flame ionization detector (8). Hexanal and *trans*-2-hexenal were identified by authentic samples, and peak areas were calibrated from authentic standards in hexane.

RESULTS

Initial experiments on the effect of volatiles generated from homogenates of 16 soybean cotyledons (approximately 2.2 g fresh weight, 1.1 g dry weight) on *A. flavus* spore germination indicated that there was no effect of homogenized soybeans alone on spore germination (Table 1). However, very little spore germination was observed when 500 units/ml of lipase was added to the soybean homogenate. Because added lipase would generate free fatty acids from triglycerides, individual fatty acids were added to soybean homogenates in the absence of lipase to determine which was responsible for the volatiles that inhibited spore germination. Neither palmitic, stearic, nor oleic acid had any affect on spore germination. In contrast, the addition of either linoleic or linolenic acid to soybean homogenates resulted in the inhibition of spore germination (Table 1). No spore germination or swelling was observed with the soybean homogenate plus linolenic acid treatment. Although some spores germinated in the soybean homogenate plus linoleic acid treatment, the germ tubes were less than 40 μm long and appeared to be thin and vacuolated in comparison to the long luxuriant growth observed in untreated cultures. In contrast to soybean cotyledons, homogenization of 32 corn germs (approximately 2.1 g fresh wt) with 500 units/ml of lipase did not generate volatiles that inhibited *A. flavus* spore germination (Table 1). As a control, the effects of 15 mM linoleic and linolenic acids in buffer on spore germination in the absence of plant homogenates were tested. Whereas 15 mm linoleic acid in buffer alone did not affect spore germination (data not shown), spore germination in the presence of 15 mM linolenic acid in buffer only was only 57% of that in the control plate, which represented a significant difference according to the Student's *t* test (data not shown).

In a series of experiments designed to test the effect of added lipase activity on the generation of volatiles that were inhibitory to fungal spore germination, increasing amounts of lipase were added to homogenates of 16 soybean cotyledons. Whereas the addition of 500 units/ml of lipase to the soybean homogenates generated volatiles that completely inhibited spore germination, the addition of activities of less than 500 units/ml of lipase resulted in less than complete inhibition of spore germination (Fig. 2). The addition of increasing lipase activity also resulted in increasing proportions of ungerminated spores. Swollen spores, intermediate to germination, increased in abundance when added lipase was increased from zero to 300 units/ml and then decreased with further increases in added lipase (Fig. 2).

The influence of the number of soybean cotyledons homogenized on the generation of volatiles inhibitory to fungal spore germination was tested in a series of experiments in which increasing numbers of cotyledons were homogenized in the presence of 1,000 units/ml of lipase (Fig. 3). Addition of only three cotyledons in the presence of 1,000 units/ml of lipase was sufficient to completely inhibit *A. flavus* spore germination. Homogenization of eight cotyledons in the presence of 1,000 units/ml of lipase resulted in complete inhibition of spore swelling as well as germination (Fig. 3).

The effect of the lipoxygenase inhibitor, NDGA, was tested for its effect on the generation of volatiles inhibitory to *A. flavus* spores from soybean homogenates in the presence of 1,000 units/ml of lipase. Four soybean cotyledons were homogenized in the presence of 1 mM NDGA (in ethanol) and incubated at room temperature for 5 min, allowing time for the inhibitor to combine with the active site of lipoxygenase. Lipase was added to the homogenate, and the effect of the generated volatiles on *A. flavus* spore germination was tested. This result was compared to lipase

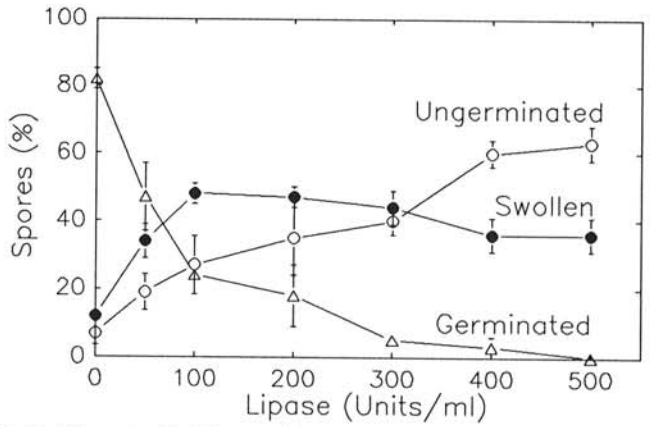

Fig. 2. Effect of added lipase activity on the generation of volatiles from soybean homogenates that inhibit *Aspergillus flavus* spore germination. All homogenates contained 16 soybean cotyledons. Data points represent means from three experiments. Error bars indicate the standard deviation.

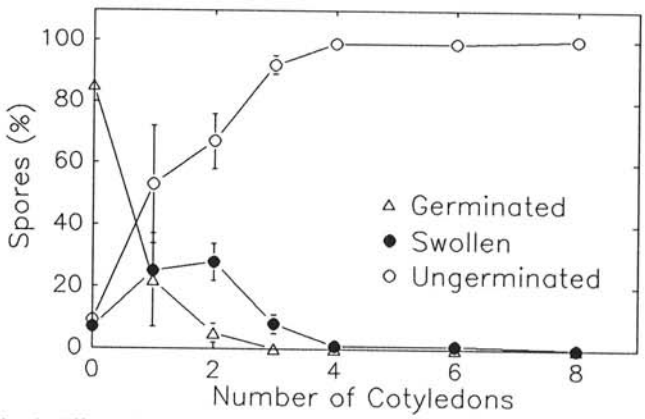

Fig. 3. Effect of the number of soybean cotyledons in the homogenate on the inhibition of *Aspergillus flavus* spore germination by volatiles generated by the homogenate. All homogenates contained 1,000 units/ml of lipase. Points represent the means from three experiments. Error bars indicate the SD.

TABLE 1. Effect of volatiles generated from soybean-cotyledon and corn-germ homogenates with various additions on the germination of *Aspergillus flavus* spores[a]

Treatment	Percent spore germination		
	Ungerminated	Swollen	Germinated
16 soybean cotyledons	6 ± 5	10 ± 2	80 ± 6
16 soybean cotyledons + 500 units/ml of lipase	81 ± 29	14 ± 20	5 ± 9
16 soybean cotyledons + 15 mM palmitic acid	9 ± 1	7 ± 4	84 ± 6
16 soybean cotyledons + 15 mM stearic acid	8 ± 4	7 ± 4	85 ± 6
16 soybean cotyledons + 15 mM oleic acid	8 ± 2	14 ± 5	78 ± 4
16 soybean cotyledons + 15 mM linoleic acid	43 ± 10	33 ± 3	24 ± 11
16 soybean cotyledons + 15 mM linolenic acid	100 ± 0	0 ± 0	0 ± 0
32 corn germs + 500 units/ml of lipase	13 ± 4	9 ± 4	80 ± 7
Buffer only	9 ± 4	9 ± 5	83 ± 9

[a] Values are the means from three experiments ± SD.

TABLE 2. The effect of the lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA), on the generation of volatiles from soybean homogenates that inhibit *Aspergillus flavus* spore germination[a]

Treatment	Percent spore germination		
	Ungerminated	Swollen	Germinated
Beans + lipase + NDGA	37 ± 4	30 ± 2	32 ± 1
Beans + lipase	68 ± 8	30 ± 6	2 ± 2
Beans only	13 ± 4	4 ± 3	88 ± 2

[a] All homogenates contained four cotyledons and were supplemented with 1,000 units/ml of lipase where indicated (total volume of 10 ml). All data are means of three replications ± SD.

plus an equivalent volume (0.1 ml) of ethanol and to soybean homogenates in the absence of lipase and inhibitor. As always, homogenized cotyledons alone did not inhibit spore germination (Table 2). Addition of ethanol with lipase into the homogenate generated volatiles that nearly inhibited spore germination completely. Addition of the lipoxygenase inhibitor, however, reduced the influence of the lipase on the generation of volatiles inhibitory to spore germination, in that only 32% of the spores germinated.

Headspace analyses of volatiles collected from soybean homogenates were performed to identify the causal agents of the inhibition of spore germination. Hexanal was identified in the headspace of untreated soybean homogenates at relatively low levels (Fig. 4). Addition of 50 and 500 units/ml of lipase to the soybean homogenates stimulated hexanal generation with time (Fig. 4), and this stimulation was more pronounced at the higher level of lipase activity. Addition of 15 mM linoleic acid to soybean homogenates in the absence of added lipase also resulted in the accumulation of high levels of hexanal in the headspace (Fig. 5A). Addition of 15 mM linolenic acid, however, resulted in the accumulation of a number of different volatile species, some of which corresponded to *trans*-2-hexenal and *cis*-3-hexenal (Fig. 5B). We noted that the apparent yield of the hexenals from linolenic acid (Fig. 5B) was reduced compared to the yield of hexanal generated from linoleic acid (Fig. 5A). This may account for our failure to consistently detect either *cis*-3-hexenal or *trans*-2-hexenal among volatiles from the headspace of lipase-treated soybean homogenate.

DISCUSSION

We demonstrated that if soybean homogenates are supplemented with either high amounts of lipase, linoleic, or linolenic acids, volatiles that will inhibit fungal spore germination are generated (Table 1). The inhibition of spore germination by volatiles from soybean homogenates appeared to be dependent on both the activity of the added lipase (Fig. 2) and on the number of cotyledons in the homogenate (Fig. 3). The generation of the volatiles also was reduced by an inhibitor of lipoxygenase (Table 2). These results indicate that the lipoxygenase pathway is involved in the generation of volatiles. Soybeans contain very high lipoxygenase activity but very low levels of free linoleic or linolenic acids (16), which are the substrates for lipoxygenase. Soybeans do, however, contain about 20% of their dry weight as triglycerides, of which linoleic acid comprises about 52% and linolenic acid about 8% of the constituent fatty acids (17). Thus, hydrolysis of soybean triglycerides by lipase should release abundant substrate for lipoxygenase, primarily as linoleic acid.

Soybeans also contain abundant activities of hydroperoxide lyase, which catalyzes the cleavage of the 13-hydroperoxide of either linoleic or linolenic acid to the C_6-aldehyde (either hexanal or *cis*-3-hexenal) and 12-oxo-*cis*-9-dodecenoic acid. We found that the addition of lipase to soybean homogenates greatly stimulated the generation of hexanal into the headspace (Fig. 4). This indicated the presence of a functional lipoxygenase pathway in the homogenate capable of generating hexanal from linoleic acid. The stimulation of hexanal evolution by the addition of linoleic acid to soybean homogenates (Fig. 5A) confirms this conclusion. Although some of the soybean lipoxygenase isozymes are capable of oxidizing triglycerides, triglyceride hydroperoxides do not serve as substrates for hydroperoxide lyase (24). Lipase-catalyzed hydrolysis of triglycerides apparently is the rate-limiting step. When free fatty acids were used as substrates, the concentration of 15 mM was arbitrarily chosen for a median value based on the triglyceride present. This would be the concentration attained if 20% of the total triglyceride was hydrolyzed to a specific fatty acid, like linoleic acid. However, 15 mM linoleic acid was probably an excess of substrate to generate the observed levels of hexanal. As indicated in Figure 5A, a maximum headspace of 145 ng/ml was attained with 15 mM linoleic acid, and this value was equivalent to the amount of hexanal that equilibrates over a 0.31 mM solution of hexanal in buffer only. Previous work showed that the addition of only 0.75 mM linoleic acid to soybean homogenates resulted in the formation of 0.2 mM hexanal (8).

Although linoleic acid in buffer alone did not inhibit fungal spore germination, linolenic acid in buffer alone caused a 43% decrease in spore germination from the control. The latter effect was attributed to autoxidation of linolenic acid to hydroperoxides followed by free-radical cleavage via an alkoxyl radical to give aldehydes. It is well-known that the oxidizability of polyunsaturated fatty acids is linearly dependent on the number of *bis*-allylic methylenes present in the fatty acid (2). Thus, linolenic acid would oxidize at roughly twice the rate of linoleic acid. According to Grosch (9), hydroperoxides from autoxidation of linolenic acid gave rise to more volatile, shorter chain aldehydes (propanal) as well as the more fungitoxic unsaturated aldehydes (2-pentenal, 2- and 3-hexenals, 2,4-heptadienal, and 2,4,7-decatrienal), compared with aldehydes derived from linoleic acid hydroperoxides (mainly hexanal and 2,4-decadienal). These results suggest that autoxidation of linolenic acid may have con-

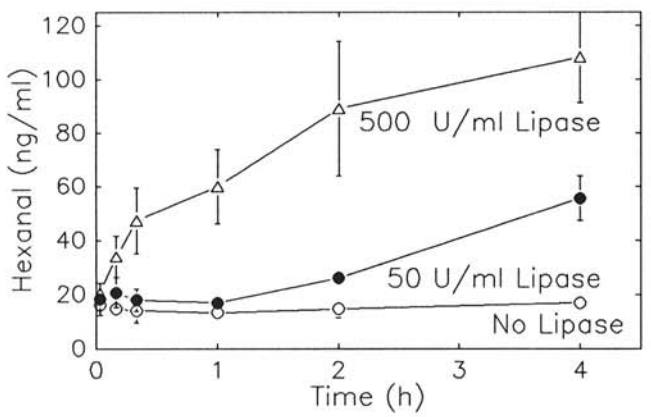

Fig. 4. Hexanal concentrations in headspace gases over soybean homogenates with 500 and 50 units (U)/ml of lipase, without added lipase activity. Values are the means from two experiments. Error bars indicate SE.

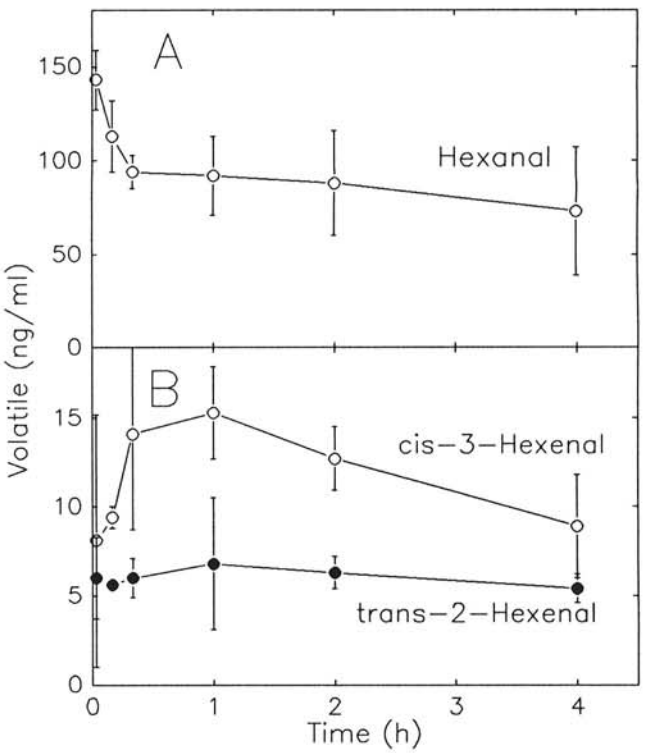

Fig. 5. A, Hexanal concentrations in headspace gases over soybean homogenates, with 15 mM linoleic added. **B,** *cis*-3-hexenal and *trans*-2-hexenal concentrations in headspace gases over soybean homogenates, with 15 mM linolenic acid added. Values are the means from two experiments. Error bars indicate SE.

tributed to the inhibition of spore germination by the soybean homogenates supplemented with linolenic acid (Table 1).

The volatiles generated from the addition of linolenic acid to soybean homogenates including *trans*-2-hexenal and *cis*-3-hexenal, generally were not observed from lipase-treated homogenates. This may be due partially to the lower abundance of linolenic as compared to linoleic acid in soybean triglycerides and to a greater partitioning of the hexenals into the aqueous phase and their metabolism into other compounds (8). Although it is likely that volatiles generated from linoleic acid were primarily responsible for the inhibition of fungal spore germination, it should be noted that *trans*-2-hexenal is about 10-fold more toxic to fungi than is hexanal (23). It is possible that low levels of these aldehydes, which may have had abundances beyond our detection limit, could have contributed to the inhibition of fungal spore germination.

The antifungal properties of hexanal and *trans*-2-hexenal are well-known (6,10,11,23). Hatanaka et al showed that crushed leaves of nearly every plant species will evolve *cis*-3- and *trans*-2-hexanal (12). The aldehyde *cis*-3-hexenal is responsible for the grassy smell generated from crushed leaves or newly mowed grass, whereas hexanal and *trans*-2-hexenal have been described as having rancid green and spicy green odors, respectively. It appears that the function of these volatiles is to inhibit infection of wounded surfaces. However, the function of lipoxygenase in soybeans beyond its function as a storage protein, is still obscure. Cytoplasmic localization of lipoxygenase separates it from any substrate and would prevent it from functioning in the intact seed (20). Furthermore, the lack of appreciable lipase activity in dry soybean seed would prevent the utilization of fatty acids stored in triglycerides as substrates for aldehyde production.

We believe that the soybean lipoxygenase pathway is triggered into activity by the secretion of lipase from invading fungi. Exogenous lipase and other secreted fungal enzymes could lyse the soybean cotyledon cells and mix their contents, as appears to occur during *A. flavus* invasion of maize sculellum (18). Lipoxygenase could then come in contact with linoleic acid released from triglycerides and react to form the 13-hydroperoxide from which hexanal would be generated by the action of hydroperoxide lyase. The hexanal would inhibit fungal growth and impede the invasion of tissue by the fungus. Thus, the destruction of the soybean cell triggers the release of aldehydes that target the invading pathogen.

Soybeans are known to be resistant to invasion by *A. flavus* and aflatoxin contamination, whereas corn germs are known to be susceptible. We demonstrated here that soybeans possess a lipoxygenase pathway that can protect them against this fungus, whereas corn germs do not possess this pathway. Although corn leaves contain the lipoxygenase/hydroperoxide lyase system (4), corn germ does not possess detectable hydroperoxide lyase. Furthermore, the lipoxygenase from corn germ is specific for the formation of the 9-hydroperoxide (7), which cannot form C_6-aldehydes. Apparently, lipid peroxidation in corn germ does not lead to appreciable hexanal generation.

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