Reduction in Fusarium Population Density in Soil by Volatile Degradation Products of Oilseed Meal Amendments

Michael A. Zakaria, J. L. Lockwood, and A. B. Filonow

Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI 48824. Present address of M.A.Z.: Department of Botany, Bogor Agricultural University, Bogor, Java, Indonesia.
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ABSTRACT

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Soybean, linseed, and cottonseed meals incorporated into a loamy sand soil at 1% concentration (w/w) reduced chlamydospore population densities of *Fusarium oxysporum* and *F. solani* from 10^5/g to 10^2/g or less after 4–6 wk incubation in closed containers. Incubation of similarly amended soil in open containers was less effective in reducing chlamydospores. No decrease in population density was observed in alfalfa meal-amended and unamended soils. Propagule densities of five additional *Fusarium* spp. incubated in soil above, but not in contact with, amended soil in closed containers also were significantly reduced. Boric acid solutions above oilseed meal-amended soils nullified the fungitoxic effect produced by soybean meal, but not that produced by linseed or cottonseed meals. The greatest amounts of titratable substances trapped in boric acid solution were produced with soybean meal, followed in order by linseed and cottonseed meals. Ammonia, ~1,000–4,000 μg/ml, was detected by multicolumn gas chromatography of the trapping solutions. Relative concentrations of ammonia were in the order: soybean meal >linseed meal >cottonseed meal. The quantity of ammonia produced was inversely related to the C:N ratios of the meals. The evidence indicates that ammonia was involved in suppression of *Fusarium* by the oilseed meal amendments, but other unidentified compounds also were involved in suppressing *Fusarium* in soils amended with either linseed or cottonseed meals. In the presence of the oilseed meals, *Fusarium* chlamydospores were first stimulated to germinate, then new chlamydospores were formed, but these were soon killed. In soil amended with alfalfa meal, chlamydospore germination also was stimulated and was followed by the formation of new chlamydospores, but these remained viable.

Linseed, cottonseed, or soybean meals incorporated into a loamy sand soil at 1% (w/w, dry weight basis) consistently reduced numbers of *Fusarium* spp. to 0.001 or less of the initial population density after 4 wk of incubation in closed containers (18). However, in spite of their effectiveness in the laboratory, the oilseed meal amendments did not reduce *Fusarium* population densities in the same soil type in the field. The purpose of this study was to investigate the mechanism by which the oilseed meals suppress selected *Fusarium* spp.

MATERIALS AND METHODS

Unless otherwise specified, techniques involving preparation of inocula, soil preparation and infestation, incorporation of organic residues into soil, estimation of *Fusarium* population densities and statistical analysis, were as previously described (18). The soil used was Oshtemo-Boyer loamy sand (18).

Fungi. In most experiments, a mixture of equal portions of wheat bran cultures of *Fusarium oxysporum* Schlect. and *F. solani* (Mart.) was used to augment *Fusarium* populations in natural soil (18).

Where *Fusarium* population densities are mentioned, this refers to total Fusaria. Five other species as recognized in the Snyder and Hansen system of classification (17) were used individually in specific experiments. These were: *F. lateritium* Nees, *F. moniliforme* Shelf., *F. rigidiusculum* (Brick) Snyd. & Hans., *F. roseum* (Lk.) Sacc., and *F. tricinctum* (Corda) Sacc. The latter five fungi were grown at 20 C in potato-maltose broth prepared by substituting maltose (20 g/L) for dextrose in potato-dextrose broth. After 4 wk, cultures were washed by centrifugation, restored to their original volumes in distilled water, and 100-ml portions were homogenized for 5 min in a Servall Omni-Mixer (Ivan Sorvall, Inc., Norwalk, CT 06856), at a rheostat setting of 50. The homogenized cultures or wheat bran cultures were individually mixed with 500 g of soil and incubated in polyethylene bags. After 8 wk, chlamydospore concentrations were constant as determined by microscopic examination and plate counts. The infested soils were air-dried and kept at 4 C until further use.

Detection of volatile inhibitors. Several experiments were designed to examine whether volatile inhibitors were involved in reducing *Fusarium* population densities in the oilseed meal-amended soils. In one experiment, duplicate 550-ml plastic pots with or without covers of polyethylene film were used for incubating 300 g of amended soil adjusted to 30% of water-holding capacity (WHC) (approximately −0.2 bars water potential). The moisture contents of the soils in the open containers were maintained by daily addition of water to bring the soils to a constant weight. In another experiment, 2-g samples of *Fusarium*-infested soil in each of four stainless steel planchets (26 mm × 7 mm) were incubated on the surface of 300 g of soil amended with oilseed or alfalfa meals, or of unamended soil, in closed 550-ml plastic pots. *Fusarium* population densities were estimated initially, and biweekly thereafter.

The presence of ammonia, amines, and other basic volatiles was detected by using 2% boric acid as a trapping solution (3,14) in glass vials placed on 300 g amended or unamended soil in triplicate closed containers. Periodically, the solutions were removed from the vials and titrated using 0.01 N sulfuric acid (3). Two-tenths ml of an indicator prepared by dissolving 0.33 g bromocresol green and 0.165 g methyl red in 500-ml ethanol was added to each vial before titration. The boric acid solutions were replenished immediately and returned to the containers of soil.

The possible nullifying effect of trapping solutions on volatile toxicity to *Fusarium* was tested. Four planchets, each containing 2 g of *Fusarium*-infested soil, and four 5.5-cm-diameter petri dishes, each containing 10 ml of 2% boric acid, distilled water, or nothing were incubated on 500 g of amended or unamended soils adjusted to 30% of WHC in triplicate closed square plastic containers (19 × 19 × 6 cm) (Fig. 1). The planchets were placed in the center, and the petri dishes at the four corners of each of the containers. Each container was enclosed in a polyethylene bag. *Fusarium* population densities were estimated at the beginning of the experiment and biweekly thereafter.

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Ammonia volatilization from oilseed meal-amended soils. Three-hundred-gram quantities of amended soils were adjusted to 30% of WHC, and were incubated in duplicate closed plastic containers (19 × 19 × 6 cm). A single small petri dish containing 10 ml of 0.01 N sulfuric acid as a trapping solution was placed on the soil surface in each container. The containers were enclosed in polyethylene bags. Aliquots (1 μl) of the trapping solution were analyzed by gas chromatography immediately following enclosure and periodically thereafter.

A Varian Series 1400 gas chromatograph (Varian Instruments, Palo Alto, CA 94303) equipped with a flame ionization detector was used for identification and quantification of ammonia and amines. A 1.83 m × 2 mm i.d. glass column, packed with Chromosorb 103, 60/80-mesh (Anspec Co., Ann Arbor, MI 48107) was held at 110 C. The carrier gas was helium at a flow rate of 30 cm^3/min. Injection and detector temperatures were 250 C and 320 C, respectively. Various concentrations of methyl-, dimethyl-, and trimethylamine standards were prepared by diluting reagent-grade aqueous solutions (Aldrich Chemical Co., Milwaukee, WI 53233) with glass-distilled water. Aqueous dilutions of reagent-grade ammonium hydroxide were used as ammonia standards. Minimum detectable amounts were 10 ng for ammonia and 2 ng for the amines.

The presence of ammonia also was confirmed by gas chromatography with a Tracor Model 560 gas chromatograph (Tracor Inc., Austin, TX 78721) fitted with a detector specific for nitrogen (Tracor Model 702 N-P). A 1.83 m × 2 mm i.d. glass column packed with 28% Pennwalt/4% KOH on 80/100-mesh Gas Chrom R (Applied Science, State College, PA 16801) was used. Oven, injector, and detector temperatures were 100, 200, and 260 C, respectively. Helium at 20 cm^3/min was the carrier gas.

The C:N ratios of the meals were calculated. Carbon content of the meals was determined with a Leco Carbon Analyzer (Leco Inc., St. Joseph, MI 49085), and nitrogen was determined by a micro-Kjeldahl method (2). A Technicon I amino acid analyzer with a single column of Chromobead C-2 (Technicon, Tarrytown, NY 10591) was used for amino acid analysis of the oilseed and alfalfa meals. Determinations were made with ninhydrin reagent, and with norleucine as the internal standard.

Observation of the fate of *F. solani* chlamydospores in soil. Chlamydospores of *F. solani* were prepared according to the method of Hsu and Lockwood (7). Conidia were germinated by incubation for 16 hr in a liquid medium containing the same ingredients as the basal portion of a *Fusarium*-selective medium (9). The germlings were aseptically separated from nongerminated conidia by filtration through a 0.15-mm (100-mesh) sieve. The retained germlings were aseptically washed several times, then suspended in sterile distilled water to obtain a density of approximately 10^3 germlings per milliliter. Two-milliliter portions of the suspension were applied onto Nuclepore membrane squares (1 cm^2, 0.4 μm pore size) by suction. The membranes were then floated on 0.03 M sodium sulfate solution for 10 days, during which time chlamydospores were formed. The membranes were buried in amended and unamended soils with a nylon net (0.4-mm mesh) separating the chlamydospores from direct contact with the soils. Triplicate 10-cm-diameter petri plates were used as the containers, and these were enclosed in small plastic bags.

Ten membranes were buried in 100-g portions of treated soil in triplicate 10-cm-diameter petri dishes. At intervals, two membranes were removed from each soil treatment, and the chlamydospores were transferred to disks of the selective agar medium to observe germination either immediately or following a 24-hr incubation period on the agar disks (15). Cotton blue in lactophenol was used to stain the chlamydospores and the germ tubes. Percentage germination was based on 100 counts from each of four different microscope fields per membrane.

All experiments were repeated one or more times with similar results.

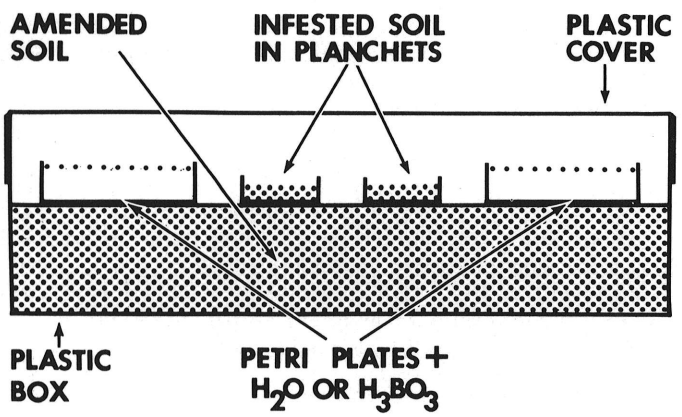


Fig. 1. Diagram of system used to test the effect of volatiles from amended soils on population densities of two *Fusarium* spp. in the presence of trapping solutions. Closed plastic box contains soil amended with oilseed meals or alfalfa meal. On the soil are placed planchets containing soil infested with chlamydospores of *Fusarium solani* and *F. oxysporum,* and glass dishes containing distilled water or 2% boric acid solution.

RESULTS

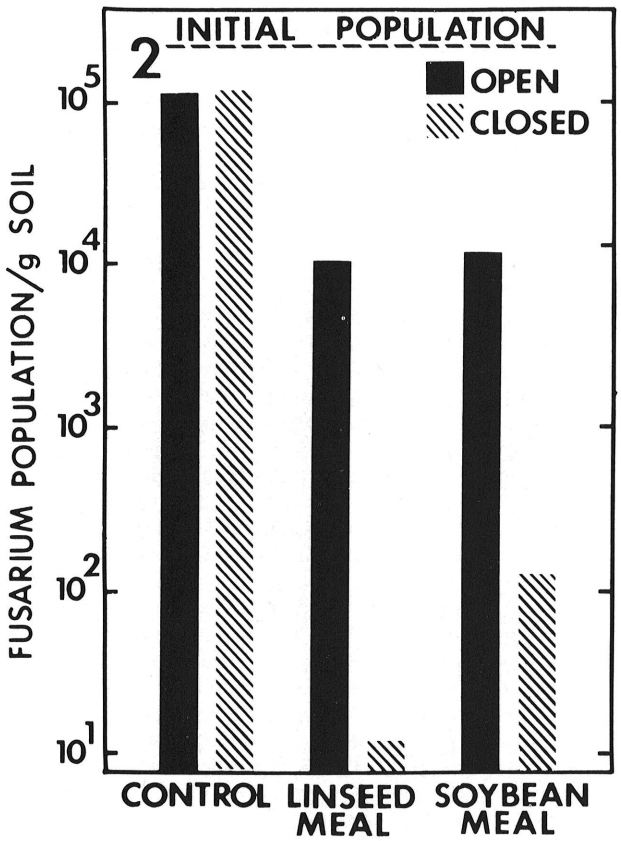
Detection of volatile inhibitors. The effect of amendments on population densities of *Fusarium* in soil incubated in open versus closed containers was compared. Amendments were 1% (w/w) alfalfa, linseed, or soybean meals (dry weight basis). Numbers of propagules in soils amended with oilseed meals and kept in closed containers were depressed more than those kept in open containers. For example, after 6 wk, propagules in soils amended with linseed and soybean meals were reduced from more than 10^5 to about 10 and 10^2/g, respectively (Fig. 2). In contrast, viable propagules in unamended soil in open containers were about 10^4/g. Propagules in alfalfa meal-amended and in the unamended control soils remained at about 10^5/g in either type of container. The much greater reduction in closed containers indicated that volatile substances might have been involved.

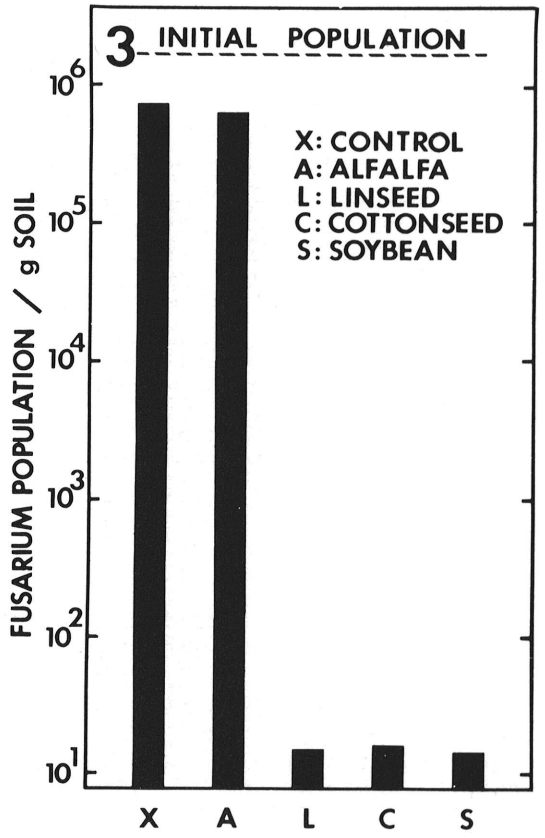
To further test this possibility, planchets containing *Fusarium*-infested soil were incubated on the surfaces of soils either unamended or amended with 1% (w/w) alfalfa, linseed, cottonseed, and soybean meals in closed containers. After 4 wk of incubation on oilseed meal-amended soils, *Fusarium* population densities in soils in the planchets had decreased to less than 0.1% of the initial level (Fig. 3), whereas population densities in soil incubated on the unamended and alfalfa meal-amended soils were not reduced. The results strongly indicated the presence of toxic volatile substances in soils amended with oilseed meals.

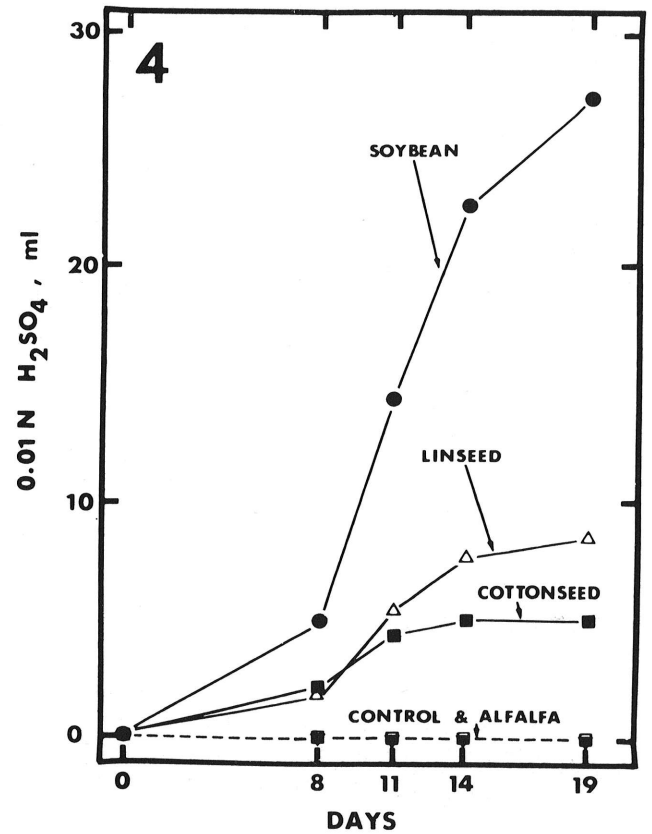
Glass dishes containing boric acid solution or distilled water were placed on the surfaces of amended or unamended soils in closed containers to trap ammonia, amines, or other basic volatile compounds (Fig. 1). The highest yield of titratable materials was derived from soil amended with soybean meal, followed in order by those amended with linseed and cottonseed meals (Fig. 4). The boric acid solutions collected from the alfalfa meal-amended and the unamended control soils did not change the color of the indicator.

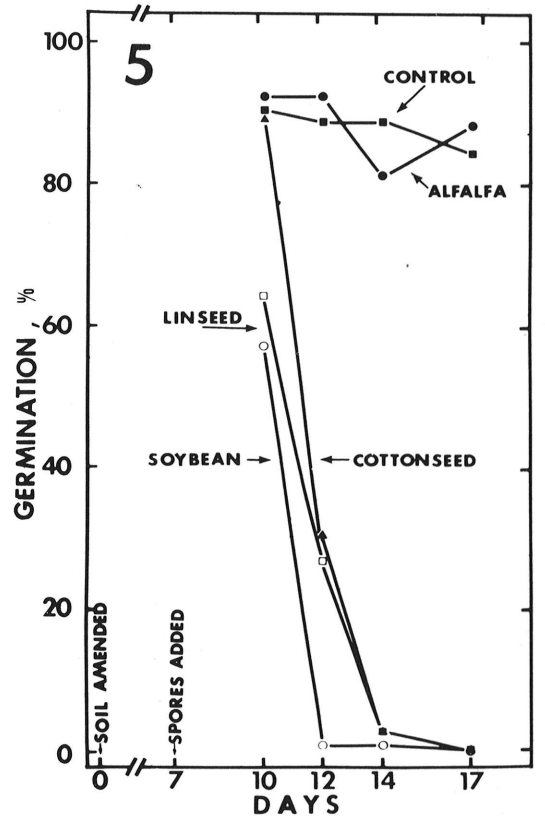
An experiment was done to examine whether the volatiles trapped by the boric acid solutions contributed to reduction in *Fusarium* propagules in soil. After 4 wk of incubation, numbers of propagules were greatly reduced in soils in planchets incubated on linseed and cottonseed meal-amended soils, regardless of the presence of the trapping solution (Table 1). However, both boric acid and distilled water prevented any decrease in *Fusarium* propagules in the presence of soybean meal-amended soil, although numbers were greatly depressed in their absence. *Fusarium* population densities in soils incubated on unamended and alfalfa meal-amended soils were not affected by the presence of the trapping solutions.

Production of ammonia. Ammonia consistently was detected by multicolumn gas chromatographic analysis of trapping solutions from oilseed meal-supplemented soils. Ammonia first was detected after 2 days of incubation of soil amended with soybean meal, and









Figs. 2–5. 2, Numbers of propagules of *Fusarium oxysporum* and *F. solani* (combined) in soil incubated in open or closed containers 6 wk after amendment with 1% (w/w) alfalfa or oilseed meals. Least significant ranges obtained by Tukey's *w* procedure for open and closed containers, were 0.17 and 0.32, respectively ($P = 0.01$). **3,** Numbers of propagules of *Fusarium oxysporum* and *F. solani* (combined) in soil samples in planchets incubated on soil amended with 1% (w/w) alfalfa or oilseed meals in closed containers for 4 wk. The least significant range obtained by Tukey's *w* procedure was 0.38 ($P = 0.01$). **4,** Titration curves of boric acid solutions incubated on the surface of soil either unamended or amended with 1% (w/w) alfalfa and oilseed meals in closed containers. **5,** Germinability of *Fusarium solani* chlamydospores in amended soils. The chlamydospores on Nuclepore membrane filters were placed in the soil 7 days after amendment with 1% (w/w) alfalfa or oilseed meal amendments. At intervals, membrane filters were removed and the chlamydospores were transferred to disks of a selective agar medium for microscopic observation of germination after 24 hr.

TABLE 1. Population densities *Fusarium* spp. in soil samples incubated in planchets on the surface of soil amended with 1% (w/w) alfalfa and oilseed meals. Petri dishes containing distilled water or 2% boric acid as trapping solutions also were placed on the soil, which was kept in closed containers for 4 wk[a]

Trapping solution	*Fusarium* propagules per gram of soil sample[a] (log_{10}, dry wt basis)				
	Unamended control	Alfalfa meal	Linseed meal	Cottonseed meal	Soybean meal
None	4.90	4.43	2.24	2.02	1.48
Water	4.74	4.51	1.74	1.48	5.00
Boric acid	4.87	4.87	2.66	1.48	5.00

[a] Initial population density (log_{10}) was 5.30 propagules per gram of natural soil. The soil had been augmented with equal portions of wheat bran cultures of *F. oxysporum* and *F. solani*.

[b] The least significant range by Tukey's *w* procedure ($P = 0.01$) for the trapping solution × soil treatment interaction was 1.38.

TABLE 2. Population densities of five *Fusarium* spp. in soil samples incubated for 4 wk in planchets on soils either unamended or amended with 1% (w/w) alfalfa and oilseed meals

Fusarium spp.	*Fusarium* propagules per gram of soil (log_{10}, dry wt basis)					
	Unamended control	Alfalfa meal	Linseed meal	Cottonseed meal	Soybean meal	LSR[a]
F. lateritium	3.91	4.06	1.40	1.40	1.25	0.24
F. moniliforme	4.62	4.44	1.55	1.55	1.10	0.32
F. rigidiusculum	4.65	4.49	1.70	1.40	1.25	0.24
F. roseum	4.12	4.35	1.40	1.40	1.10	0.08
F. tricinctum	4.14	4.38	1.55	1.55	1.25	0.39

[a] Least significant range (LSR) values were obtained by applying Tukey's *w* procedure ($P = 0.01$).

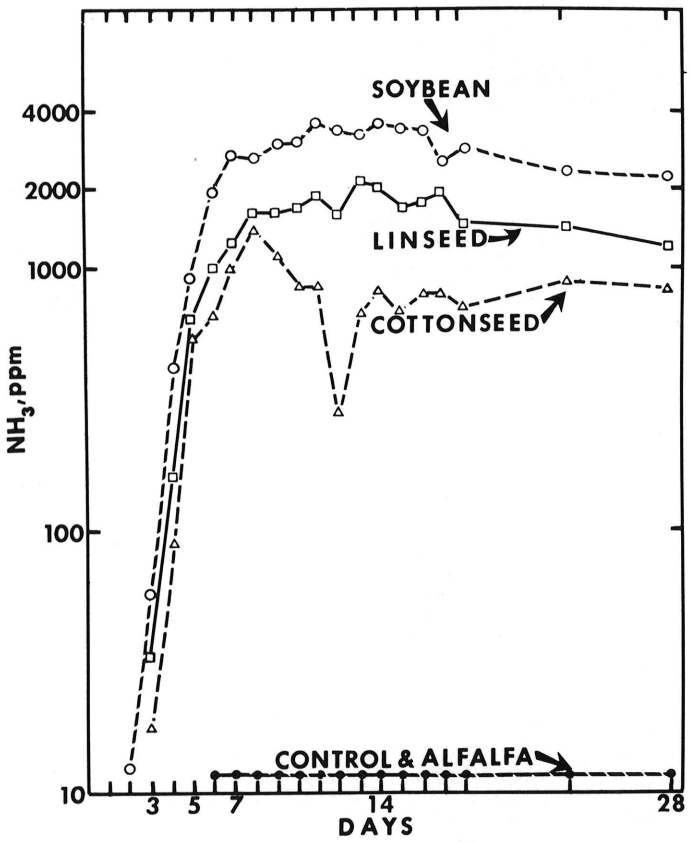


Fig. 6. Ammonia concentrations in trapping solutions incubated on the surface of soils either unamended or amended with 1% (w/w) alfalfa and oilseed meals in closed containers, as determined by gas chromatography.

after 3 days of incubation with linseed and cottonseed meals (Fig. 6). Thereafter, ammonia production increased rapidly until about the fifth day, when rates of production declined. Peak ammonia production occurred after 8 days of incubation (1,394 μg/ml) for cottonseed meal, 11 days (3,747 μg/ml) for soybean meal, and 13 days (2,187 μg/ml) for linseed meal. Throughout the experiment, ammonia production by oilseed meal-amended soils followed the order: soybean meal > linseed meal > cottonseed meal. After 28 days of incubation, the ammonia concentrations in the trapping solutions were 2,468, 1,225, and 825 μg/ml respectively. Ammonia was detected in the alfalfa-amended and unamended soils only after 6 days of incubation. Throughout the remaining incubation period, ammonia production from these soils was detectable, but at levels too low for quantification (~10 μg/ml). Amines were not detected.

The pH of the trapping solutions rose from 2.0 before incubation to 9.1, 8.6, and 7.4 after 28 days for solutions above soils amended with soybean, linseed, or cottonseed meals, respectively. There was no pH change in solutions above alfalfa-amended and unamended soil.

In an attempt to explain the differences in ammonia production among the meals, their amino acid compositions were determined. Amino acid analyses showed no singular differences for soybean meal, as compared with linseed and cottonseed meals, that would account for the higher production of ammonia from soil amended with soybean meal. However, all three meals contained nearly three times more total amino acids than did alfalfa meal (w/w). The C:N ratios of the meals were 6.7 for soybean meal, 7.2 for linseed meal, 7.4 for cottonseed meal, and 17.1 for alfalfa meal. The C:N ratios, therefore, may be related to the capacities of the amendments to produce ammonia.

The fate of *Fusarium* chlamydospores in the amended soils. To study the mechanism of reduction of *F. solani* propagules in the oilseed meal-amended soils, chlamydospores of *F. solani* were placed on membrane filters and buried in soils amended with oilseed meals, alfalfa meal, or in unamended soil, for different lengths of time.

In one experiment, the chalamydospores were buried at the same time as the soils were amended. When the membranes were recovered and examined 24 hr after burial, 95% or more of the chlamydospores from all amended soils had germinated, but none had germinated in the unamended soil. Production of conidia and some lysis of hyphae had occurred in all amended soils by 48 hr after burial. At 3 days, lysis of hyphae was very extensive and production of new chlamydospores was frequently seen in the amended soils. When membranes were removed from amended soils 7–21 days after burial, many new chlamydospores were present, but none had germ tubes. No hyphae were present. Viability of chlamydospores was tested by incubating recovered membranes on a selective agar medium. After 7 days of incubation in the oilseed meal-amended soils, fewer than 30% of the chlamydospores had germinated after 24 hr of incubation on the agar disks, whereas germinability of the chlamydospores buried in the unamended and the alfalfa meal-amended soils was greater than 80%. After 21 days of incubation in the oilseed meal-amended soils, the germinability of the chlamydospores was completely lost, while in the unamended and the alfalfa meal-amended soils, 65% or more of the chlamydospores were still viable. In another experiment, the chlamydospores were buried in the soils 7 days after soil amendment. After 7 days of incubation, the germinability of the chlamydospores in the oilseed meal-amended soils was nearly zero, whereas those incubated in the unamended and the alfalfa meal-amended soils showed 80% or more germination (Fig. 5). Similar results were obtained when the membranes were removed from the treated soils after 10 days.

Effect of oilseed meal amendments on five additional *Fusarium* spp. Two-gram portions of soil infested with either *F. lateritium, F. moniliforme, F. rigidiusculum, F. roseum,* or *F. tricinctum* were placed in planchets. Four planchets of each species were incubated on the surface of 500 g of soil either unamended or amended with 1% (w/w) alfalfa, soybean, linseed, or cottonseed meal in closed containers (19 × 19 × 6 cm).

After 4 wk of incubation, viable propagule densities of all five

Fusarium spp. had decreased greatly on the oilseed meal-amended soils (Table 2). There was no decrease on the unamended or the alfalfa meal-amended soils.

DISCUSSION

Linseed, cottonseed, and soybean meals incorporated into a loamy sand soil at a rate of 1% (w/w) effectively reduced population densities of *F. oxysporum* and *F. solani* to 0.001 or less of the original level, in closed containers in the laboratory (18). Further work reported here showed that *Fusarium* propagules decreased much more in oilseed meal-amended soils incubated in closed containers than in open containers. Large decreases in *Fusarium* population densities also were obtained in soil placed in planchets and incubated on the surfaces of oilseed meal-amended soils in closed containers. These results provide strong evidence that volatile inhibitory substances were involved. The volatility of the toxic substances may explain why the oilseed meal amendments were ineffective in field experiments (18). The experimental plots were not covered, and the volatile substances, if produced, may have escaped.

The volatility of the toxic substances may limit the practical use of the oilseed meal amendments to control diseases caused by *Fusarium* spp. However, this may be compensated in part by their broad range of effectiveness against different *Fusarium* spp. and the magnitude of the reduction achieved. Moreover, substantial reduction in propagules of *Fusarium* spp. was obtained at amendment rates as low as 0.25% for soybean meal and 0.5% for linseed and cottonseed meals, after 6 wk of incubation. Whether oilseed meal amendments will effectively reduce numbers of propagules of other pathogenic fungi is still to be investigated.

The identification of ammonia by gas chromatography of trapping solutions above oilseed meal-amended, enclosed soils, strongly implicate this compound in the observed reductions in *Fusarium* population densities. Ammonia has been shown to be a fungistatic volatile emanating from alkaline soils (8,12) or soils to which lime was added to elevate soil pH (12). In addition, ammonia was shown to be a fungistatic volatile in chitin-amended soil (14), and was presumed to be one of the volatiles that affected the behavior of *Rhizoctonia solani* in soil amended with various plant residues (10). In soil amended with soybean meal, the ammonia trapped in boric acid or water apparently was solely responsible for the reduction in numbers of *Fusarium* spp. propagules, at least insofar as volatile activity is concerned, since toxicity was nullified by the presence of those solutions. The fungitoxic effects of similarly treated linseed and cottonseed meal-amended soils, however, were not nullified, suggesting that other volatile toxic substances also may have evolved from soil containing these amendments.

Soil amended with soybean meal consistently produced more ammonia than soil amended with linseed or cottonseed meals. In most experiments, amendment of soil with soybean meal also more effectively reduced chlamydospore populations than did amendments with linseed or cottonseed meals (18). The greater ammonia production in soil amended with soybean meal may have accounted for its enhanced effect. The greater production of ammonia by soybean meal in soil could not be explained by the amino acid composition of the amendments, but appeared to be related to the C:N ratios of the meals, though the differences were not great. Alfalfa meal, which proved to be an ineffective amendment in this work, had a C:N ratio more than twice as large as the oilseed meals. Lewis and Papavizas (10) also showed a close association of amendments with low C:N ratios with production of volatile inhibitors, which presumably were ammonia and/or amines. In their study, immature corn (C/N = 9/1), rye (C/N = 9/1), and buckwheat (C/N = 16/1) were generally more effective in reducing survival and saprophytic activity of *Rhizoctonia solani* in soil than were mature residues (C/N = 48/1 to 94/1).

Amendment of soil with either alfalfa or oilseed meals temporarily nullified the fungistatic property of the soil. In freshly amended soils, 95% or more of *Fusarium* chlamydospores on buried membranes germinated after 1 day, while no germination occurred in unamended soils. Germination was followed by production of conidia, lysis of hyphae, and production of new chlamydospores during the first 3 days of burial. Fungistasis of the soils, however, was restored within 7 days after incorporation of amendments: no germination of chlamydospores was observed in soil after this period. Continued incubation in oilseed meal-amended soils led to a progressive attenuation of chlamydospore viability. In contrast, viability of the chlamydospores in alfalfa meal-amended and unamended control soils remained high even after 21 days of burial. These results indicated that the mechanism of propagule reduction was not germination-lysis as has been reported in several cases (1,4–6,11,16), but rather, a direct killing of the newly formed, but ungerminated chlamydospores by ammonia, or other toxic volatile compounds, produced during degradation of the oilseed meals (13).

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