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.

We thank Sharon Mohrlok for the amino acid analysis. Journal Series Article 9111, Michigan Agricultural Experiment Station. Accepted for publication 24 October 1979.

ABSTRACT


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 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 multicolour 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.

**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 Schlecht. 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 Shellef., F. rigidusculum (Brick) Snyder & 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 X 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 X 19 X 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.

©1980 The American Phytopathological Society
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 cm³/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 Varian Model 560 gas chromatograph (Varian Inc., Austin, TX 78721) fitted with a detector specific for nitrogen (Tracer Model 702 N-P). A 1.83 m × 2 mm i.d. glass column packed with 28% Perminvar, 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 cm³/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 Chromosbread 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 norelucine 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 level (Fig. 3), whereas population densities in soil incubated on the agar disks (15) were placed on the surfaces of amended or unamended soils in planchets had decreased to less than 0.1% of the initial level (Fig. 3), whereas population densities in soil incubated on the agar disks remained at about 10⁵/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, or soybean meals. After 4 wk of incubation on 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 10⁵ to about 10⁴/g, respectively (Fig. 2). In contrast, viable propagules in unamended soil in open containers were about 10⁴/g. Propagules in alfalfa meal-amended and in the unamended control soils remained at about 10⁵/g in either type of container. The much greater reduction in closed containers indicated that volatile substances might have been involved.

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.

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

![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.](image-url)
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 test 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 test 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 of 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 were also placed on the soil, which was kept in closed containers for 4 wk.

<table>
<thead>
<tr>
<th>Trapping solution</th>
<th>Unamended control</th>
<th>Alfalfa meal</th>
<th>Linseed meal</th>
<th>Cottonseed meal</th>
<th>Soybean meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.90</td>
<td>4.43</td>
<td>2.24</td>
<td>2.02</td>
<td>1.48</td>
</tr>
<tr>
<td>Water</td>
<td>4.74</td>
<td>4.51</td>
<td>1.74</td>
<td>1.48</td>
<td>5.00</td>
</tr>
<tr>
<td>Boric acid</td>
<td>4.87</td>
<td>4.87</td>
<td>2.66</td>
<td>1.48</td>
<td>5.00</td>
</tr>
</tbody>
</table>

*Initial population density (logio) 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.

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.

<table>
<thead>
<tr>
<th>Fusarium spp.</th>
<th>Unamended control meal</th>
<th>Alfalfa meal</th>
<th>Linseed meal</th>
<th>Cottonseed meal</th>
<th>Soybean meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. lateritum</td>
<td>3.91</td>
<td>4.06</td>
<td>1.40</td>
<td>1.25</td>
<td>0.24</td>
</tr>
<tr>
<td>F. moniliforme</td>
<td>4.62</td>
<td>4.44</td>
<td>1.55</td>
<td>1.10</td>
<td>0.32</td>
</tr>
<tr>
<td>F. rigidisculum</td>
<td>4.65</td>
<td>4.49</td>
<td>1.70</td>
<td>1.40</td>
<td>1.25</td>
</tr>
<tr>
<td>F. roseum</td>
<td>4.12</td>
<td>4.35</td>
<td>1.40</td>
<td>1.10</td>
<td>0.08</td>
</tr>
<tr>
<td>F. tricinctum</td>
<td>4.14</td>
<td>4.38</td>
<td>1.55</td>
<td>1.25</td>
<td>0.39</td>
</tr>
</tbody>
</table>

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

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 chlamydospores 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, 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, 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. lateritum, F. moniliforme, F. rigidisculum, 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 implicates 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 citrin-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 fungistic 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).

**LITERATURE CITED**