Ammonia and Nitrous Acid from Nitrogenous Amendments Kill the Microsclerotia of *Verticillium dahliae*

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ABSTRACT

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This study examined the mechanisms by which nitrogenous amendments such as meat and bone meal kill the soilborne plant pathogen *Verticillium dahliae*. The effect of nitrogen products from the amendments on the survival of microsclerotia of *V. dahliae* was examined by solution bioassay and soil microcosm experiments. Ammonia and nitrous acid but not their ionized counterparts, ammonium and nitrite, were toxic to microsclerotia in bioassays. In microcosms, addition of meat and bone meal (2.5%) to an acidic loamy sand resulted in the accumulation of ammonia and death of microsclerotia within 2 weeks. At lower concentrations (0.5 and 1%), microsclerotia were killed after 2 weeks when nitrous acid accumulated (>0.03 mM). In an alkaline loam soil, micro-

sclerotia survived at 3% meat and bone meal and neither ammonia nor nitrous acid accumulated. The toxicity of ammonia to the pathogen was verified by increasing the concentration of meat and bone meal to 4% or addition of urea (1,600 mg of N per kg) to the loam soil resulting in the accumulation of ammonia (>35 mM) and death of microsclerotia. The toxicity of nitrous acid was verified by adding ammonium sulfate fertilizer to an acidic sand soil. Inhibiting nitrification with dicyandiamide revealed that nitrous acid was generated as a result of the accumulation of nitrite and an acidic pH. Thus, levels to which the toxins accumulated and the effective concentration of amendment were dependent upon the soil examined. Of the two mechanisms identified, accumulation of nitrous acid is the more promising strategy to control plant diseases in acidic soil because it is more toxic than ammonia and is formed at lower concentrations of amendments.

Nitrogenous organic amendments ($>1,600$ kg of N per ha or ≈ 20 t/ha) including proteinaceous materials, chitin-derived materials, animal manures, and plant residues (2,3,18,27,28,49) as well as ammonia/ammonium-based fertilizers (>400 kg of N per ha) have reduced populations of soilborne plant pathogens and pests (11,12,24,35). However, the concentrations at which those materials were effective are not practical because of their excessive cost (56) and loading of nitrogen in soil, difficulty of application, and potential phytotoxicity to crops (14,31,48). The goal of this study was to determine the mechanisms by which nitrogenous amendments kill pathogens as a first step toward reducing the concentration of amendments needed.

The accumulation of ammonia (NH_3) is the mechanism most often implicated in killing pathogens in soil treated with nitrogenous amendments. Several plant pathogens have been killed at elevated levels of ammonium and soil pH (12,21,39); conditions for the formation of ammonia. Treatments killing pathogens have also given rise to the accumulation of ammonia estimated from levels of soil ammonium and pH (14,31,60) or measured directly in soil gas (9).

Tsao and Oster (60) advanced that in addition to ammonia, the accumulation of nitrous acid (HNO_2) killed *Phytophthora cinnamomi* and *P. parasitica* in amended soil. Concentrations of ammonia at 1 week and nitrous acid at 2 weeks in an acidic sandy loam soil amended with poultry manure (2%) or urea (0.1%) were

lethal to the pathogens in solution. Other pathogens and pests have been killed in solutions of nitrous acid (10,39,41,47,66).

The effect of concentration and persistence of ammonia or nitrous acid in soil on pathogen survival is unknown. Tsao and Oster (60) relied on the results of solution bioassays to predict pathogen survival in soil, but concentrations of ammonia lethal to aquatic organisms in solution bioassays and in situ differed (25). In their study, Tsao and Oster (60) compared concentrations of ammonia as milligram of N per kilogram of soil with milligram of N per liter in solution bioassays. Soil consists of solids and water, thus the expression of ammonia on a mass basis of soil does not reflect the concentration in soil solution in contact with *Verticillium dahliae* Kleb. microsclerotia. Many investigators, including Tsao and Oster (60), have used 2 M KCl extracts to estimate ammonium plus ammonia in soil. However, ammonium and ammonia held to nonprotonated and protonated cation exchange sites, respectively, are displaced into solution by potassium cations (30), resulting in a twofold over-estimation of ammonia in the solution of soil (55). Although a 2 M KCl extract reflects the availability of the nutrients to crops, it cannot be used to estimate levels of ammonia in soil without accounting for displacement of ammonium and ammonia from exchange sites. The studies of Conn and Lazarovits (14) and Lazarovits et al. (31) are the only ones, to our knowledge, reporting that the death of *V. dahliae* coincided with the accumulation of ammonia in the same soil. However, in those studies, germination of microsclerotia of *V. dahliae* was determined at 4 weeks and the timing of ammonia accumulation could not be directly linked to the death of the pathogen.

The means of demonstrating that ammonia or nitrous acid, and not other mechanisms, kill pathogens is lacking. We have taken the approach that ammonia or nitrous acid toxicity is implied if the death of a pathogen coincides with the accumulation of either

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compound in soil. Furthermore, the death of a pathogen when exposed to similar concentrations of ammonia or nitrous acid in the soil solution and solution bioassays supports their role as fungitoxic compounds.

The objective of this study was to determine if ammonia and nitrous acid toxicity are responsible for nitrogenous amendments killing the microsclerotia of *V. dahliae*. The effect of pH and the ionized (ammonium and nitrite) and nonionized (ammonia and nitrous acid) forms of the compounds were examined in solution bioassays. The survival of microsclerotia in response to ammonia and nitrous acid generated from meat and bone meal (0 to 2.5%) added to an acidic sandy loam was examined in microcosms. Confirmation that nitrous acid is toxic in soil was undertaken by adding ammonium sulfate and, conversely, by inhibiting nitrification to prevent the accumulation of nitrous acid in an acid sand. The toxicity of ammonia in soil was verified by adding a higher concentration of meat and bone meal (4%) to an alkaline loam in which the amendment was not effective up to 3% addition. Furthermore, urea (0 to 1,600 mg of N per kg) was added to the alkaline loam to specifically generate ammonia. Preliminary results of this study have been presented (32,57).

MATERIALS AND METHODS

A single-spore isolate of *V. dahliae* was obtained from an eggplant (*Solanum melongena* L. cv. Imperial Black Beauty) grown in soil from a commercial potato (*Solanum tuberosum* L.) field near Alliston, Ontario (denoted site B). The isolate was grown in semi-solid Czapek-Dox medium for 3 weeks in the dark at 24°C (22). The culture was poured through mesh screens to obtain microsclerotia of 76 to 106 µm in diameter (22). The microsclerotia were stored in the dark at 24°C prior to use.

Effect of pH, ammonium, and ammonia. Factorial treatments of soil pectate tergitol agar (22) were prepared to concentrations of 0, 25, 50, 100, and 200 mM ammonium chloride and at pH 7, 7.6, 8, and 8.5 (±0.03). A portion of the medium of each treatment was added to fill three petri dishes. The pH of the solid medium in the dishes was determined with ColorpHast pH 5 to 10 strips (EM Science, Gibbstown, NJ). Twenty-five microsclerotia were transferred to each plate by a sterile hypodermic needle. The plates were wrapped with Parafilm and incubated for 2 weeks in the dark at 24°C. The pH of the medium was determined as well as the viability of microsclerotia as the percentage of microsclerotia that germinated to form colonies. This and each subsequent experiment reported were performed twice, with three replicates each.

Effect of pH, nitrite, and nitrous acid. Factorial treatments of citric acid-NaOH 0.02 M buffered solutions (43) were prepared to final concentrations of 0, 3.38, 6.75, 13.5, 27, and 54 mM sodium nitrite and at pH 4, 5, and 6. Each solution was filter-sterilized (<0.22 µm), and 15 ml was added to three autoclaved screw-cap tubes of 15.5-ml capacity each.

Microsclerotia encased in a polyester mesh bag were added to the solution in each tube. Mesh bags were prepared from polyester screening (Saatile High Tech Fabric; 48 µm pore size; Saati S.p.A., Como, Italy) to dimensions of approximately 15 × 20 mm. Crushed silica sand was passed through mesh screens to obtain sorted particles of diameter similar to microsclerotia (75 to 106 µm). Microsclerotia (15 mg) were added to the sorted sand particles (5 g), approximately 25 mg of this mixture was added to a bag, and the bag was sealed. The tubes were incubated in the dark at 24°C for 1 day. The pH and the concentration of nitrite in each solution were then determined. Mesh bags were retrieved from solutions, rinsed with sterile distilled water, and dried at room temperature for 3 h.

Microsclerotia were dispersed onto agar medium. The lid of a soil pectate tergitol agar plate was removed, and the plate was placed on the base of a cascade impactor (Andersen Instruments

Inc., Smyrna, GA). Sieves of 0.81- and 1.18-mm-pore diameters and a conical inlet were stacked upon the base of the impactor so the whole unit housed the agar plate. A pipette bulb was inserted into the sample inlet, and suction was applied to the base of the unit. Microsclerotia were distributed onto the plate by pouring the contents of a mesh bag onto the inlet, and the bulb was quickly removed and inserted repeatedly. Tapping the sieves and inlet several times followed by cleaning with a paintbrush and laboratory tissue prevented the carryover of microsclerotia between platings of mesh bags.

The agar plates were incubated for 2 weeks in the dark at 24°C, and the microsclerotia were examined microscopically. A sectioned grid (0.25 cm²) was placed on the base of a dissecting microscope. A plate was placed on the grid and scanned systematically at ×20 magnification until 50 microsclerotia had been examined and scored for the formation of colonies. The viability of microsclerotia was determined as the percentage of the 50 microsclerotia examined that germinated to form colonies. Occasionally, microsclerotia were positioned too close to one another to differentiate, which led to the formation of a colony. These microsclerotia were excluded from examination. Also excluded from examination were microsclerotia smaller than sand grains. These microsclerotia were smaller than 76 µm in diameter and became nonviable within several weeks of being obtained from culture (22).

Effect of duration of exposure. The effect of the duration of exposure to solutions of ammonia or nitrous acid on the germination of microsclerotia was examined. Glycine-NaOH solutions at pH 8.6 were prepared to a final concentration of 0, 3.38, 6.75, 13.5, 27, and 54 mM ammonium chloride. Citric acid-NaOH solutions at pH 5 were prepared to a final concentration of 0, 3.38, 6.75, 13.5, 27, and 54 mM sodium nitrite. Each solution was filter-sterilized and added to tubes. A mesh bag containing microsclerotia was submerged in the solution in each tube. The tubes were closed and incubated in the dark at 24°C. The germination of microsclerotia was determined after 8, 24, and 96 h of incubation. A maximum exposure of 96 h was chosen because microsclerotia germinated after this time.

After 96 h of incubation, the pH and the concentration of ammonium plus ammonia or nitrous acid plus nitrite in solutions were determined. The effect of the ionic strength of solutions on the germination of microsclerotia was determined by the addition of sodium chloride at concentrations similar to those used for ammonium chloride in glycine-NaOH or sodium nitrite in citric acid-NaOH solutions.

Effect of atmosphere above solutions. Solutions of glycine-NaOH at pH 8.6 and citric acid-NaOH at pH 5 containing ammonium chloride and sodium nitrite, respectively, were prepared as previously described. Three standard-mouth mason jars (250 ml) received 50 ml of each solution. A mesh bag containing microsclerotia was suspended in the atmosphere of each jar. The jar was closed to create a sealed environment, incubated for 4 days in the dark at 24°C, and the germination of microsclerotia was evaluated.

Nitrous acid is not a gas, but decomposes to form the gases nitric oxide (NO) and nitrogen dioxide (NO₂) (40). The concentration of nitric oxide, nitrogen dioxide, and another nitrogen oxide gas, nitrous oxide (N₂O), in the headspace above solutions of nitrous acid was determined. Citric acid-NaOH solutions at pH 5 were prepared to a final concentration of 0, 0.029, and 0.117 mM nitrous acid, and 50 ml of each solution was added to three jars. The jars were closed and incubated for 1 day in the dark at 24°C. Gas samples (1 ml) were extracted by syringe from the headspace of jars and analyzed for nitrogen oxide and nitrogen dioxide by the chemiluminescent reaction of nitrogen oxide with ozone by a NO/NO₂/NO_x analyzer (Model 42; Thermo Environmental Instruments, Franklin, MA). Gas samples (2 ml) were analyzed for nitrous oxide by a gas chromatograph (Model 5880 A; Hewlett

Packard, Avondale, PA) equipped with an electron capture detector and Porapak Q column.

Soils. Soil samples were collected from two fields (sites B and M) planted with potato (*Solanum tuberosum* L.) located near the town of Alliston, Ontario, and a field planted with corn (*Zea mays* L.) at the Agriculture and Agri-Food Canada research farm located in London, Ontario (site L). Samples were collected from five locations (each $0.25 \times 0.15 \text{ m}^2$) within a site. The samples from a site were combined and stored at 5°C . A portion of the combined sample of soil was air dried and passed through a 2-mm mesh screen just prior to use. The use of screened air-dried soil facilitated the loading of soil into test tubes, ensured uniform packing of soil among replicate tubes and experimental repeats, and improved soil homogeneity between smaller samples obtained from tubes for chemical analyses. Site B soil is an acidic sandy loam, site M an acidic sand, and site L an alkaline loam soil (Table 1).

Addition of meat and bone meal and inhibition of nitrification in site B soil. The effect of accumulation of ammonia and nitrous acid in meat and bone meal-amended soil on the germination of microsclerotia was examined in microcosms. Meat and bone meal was obtained from Rothsay Industries (Dundas, ON). It had a pH of 6.5 in water, 3% moisture, 38.4% organic carbon, and 7.7% organic nitrogen. Meat and bone meal was added to duplicate polyethylene bags containing 500 g of air dried site B soil to concentrations of 0, 0.5, 1, 2, and 2.5% and thoroughly mixed. One bag at each concentration of meat and bone meal was amended with dicyandiamide (Cyanoguanidine, Sigma Chemical Co., St. Louis) at 360 mg/kg of oven-dried soil. Dicyandiamide was added to prevent nitrification and thereby determine the role nitrous acid has in killing microsclerotia. To ensure a uniform distribution throughout the soil, dicyandiamide was added in a 1:1 mixture with purified powdered talcum (Fisher Scientific Co., Nepean, Ontario, Canada). The other duplicate had talcum alone added.

Soil microcosms were prepared by adding subsamples (20 g) of the mixtures to glass test tubes ($22 \times 150 \text{ mm}$). One mesh bag containing microsclerotia was buried in the soil, and another was wedged approximately 3 cm above the soil surface. The microsclerotia above the soil served to examine the presence of volatiles toxic to the pathogen. Two aliquots of distilled water was added to bring the moisture content of the soil to -0.033 MPa . This soil moisture content was chosen to provide conditions between 50 and 60% water-filled pore space and ensure the activity of soil microorganisms was not limited by moisture (33). The tubes were covered with plastic caps that allowed for the exchange of air. All tubes were incubated in the dark at 24°C . Water was added to the tubes at weekly intervals to replace that lost by evaporation. At day 0, and after 4, 7, 10, 14, 21, and 28 days of incubation, germination of microsclerotia and chemical analyses of three tubes from each treatment were performed.

Inhibition of nitrification in ammonium sulfate-amended site M soil. The effect of nitrous acid on the germination of microsclerotia was specifically examined by addition of ammonium

sulfate to another acidic soil, site M, at concentrations of 0, 200, and 400 mg of N per kg of oven-dried soil, with or without dicyandiamide. One mixture of 500 g of air-dried soil in a polyethylene bag was prepared for each treatment and distributed to test tubes. Dicyandiamide was added at 120-mg/kg oven-dry soil the first time this experiment was performed and increased to 360-mg/kg soil in the repeat. The concentration of dicyandiamide was increased to completely prevent nitrification. The germination of microsclerotia retrieved from soil and chemical analyses were determined at day 0, and after 1, 2, 3, 4, and 6 weeks of incubation.

Addition of meat and bone meal to site L soil. The effect of meat and bone meal (0, 1, 2, 3, and 4% mass of oven-dry soil) on the accumulation and toxicity of ammonia and nitrous acid in an alkaline loam soil, site L, was examined. The germination of microsclerotia retrieved from soil and chemical analyses were performed on day 0, and after 1, 2, 3, and 4 weeks of incubation.

Addition of urea to site L soil. The effect on germination of microsclerotia of a synthetic nitrogenous fertilizer, urea, which specifically produces ammonia in soil was examined. Urea decomposes in soil to produce ammonia and carbon dioxide. Reagent grade urea was added to site L soil at concentrations of 0, 0.09, 0.18, 0.27, and 0.36%. The urea was powdered and dried (70°C for 24 h) prior to being added to soil. The treatments of urea were equivalent to the amount of nitrogen added to soil with meat and bone meal at concentrations of 0, 0.5, 1, and 2%. The germination of microsclerotia retrieved from soil and chemical analyses were performed at day 0, and after 1, 2, 3, and 4 weeks incubation.

Chemical analyses of soil microcosms and bioassay solutions. On a day of analysis, the mesh bags containing microsclerotia were removed from soil microcosms. The soil in the tube was mixed with forceps. A subsample (8 g) was added to 40 ml of cold distilled water placed in a polyethylene sack ($17 \times 30 \text{ cm}$). The sack was sealed and the slurry was mechanically disrupted with a Stomacher laboratory blender (Seward Medical, London, UK) for 30 s at normal speed and the sacks were shaken for 1 h (200 rpm) at 4°C . They were removed from the cold and placed at room temperature. The slurries were again mechanically disrupted and allowed to stand and reach room temperature.

The pH of the slurry or bioassay solutions was determined. A 1-ml aliquot of the soil suspension was centrifuged ($18,000 \times g$ for 5 min) at 4°C . The bioassay solution or cleared supernatant (300 μl) of the suspension was placed into a plastic conical insert and enclosed into a 4-ml screw-cap vial. The vial was kept at 4°C . The concentration (milligram of N per liter) of ammonium plus ammonia, nitrous acid plus nitrite, and nitrate in solution were measured by chemical suppression ion chromatography and conductivity detection (Dionex Model 100; Dionex Corp., Sunnyvale, CA) (31). Supernatant and bioassay solutions contained in vials were introduced (12.5 μl) to the ion chromatograph by an auto-sampler equipped with a refrigerated chamber to house the vials (Waters 717plus; Waters Associates, Milford, MA). The concen-

TABLE 1. Characteristics of the soils used in this study^a

Soil	pH ^b	% Organic C ^c	% N ^d	CEC ^e	-0.033 MPa ^f	% Sand ^g	% Silt ^g	% Clay ^g	Texture
Site B	5.35	1.18	0.10	2.7	0.19	71	23	6	Sandy loam
Site L	8.05	2.06	0.15	19.3	0.25	43	41	16	Loam
Site M	5.50	0.86	0.04	4.1	0.16	90	6	4	Sand

^a All analyses were performed by M. Tenuta except those for % N and cation exchange capacity (CEC) which were determined by the Soil and Nutrient Laboratory of the University of Guelph, Ontario.

^b pH of a 1:5 (soil/water) soil suspension.

^c Organic carbon content was determined by the Walkely-Black oxidation method (58).

^d Total nitrogen content was determined by the Dumas oxidation method with a LECO CNS-1000 analyzer (LECO Corp., St. Joseph, MI).

^e CEC was measured as cmol^+/kg of soil (at 105°C for 24 h) and sum of BaCl_2 extractable cations (Ca, Mg, K, Na, Al, Fe, and Mn) (23).

^f Moisture content was measured as mass of water/mass of oven-dry soil done by water desorption of saturated soil cores at -0.033 MPa with a pressure plate apparatus (59).

^g Particle size distribution was determined by the hydrometer method with pretreatment of soil to remove organic matter and carbonate minerals (52).

tration of calibrants ranged from 0 to 100 mg of N per liter for ammonium and nitrate, and either 0 to 5 or 0 to 50 mg of N per liter for nitrite depending upon the concentration in samples. The concentrations of all ions in bioassay solutions and in the soil solution are reported in millimolar of soil or bioassay solution. The concentration of ammonia and nitrous acid were estimated with the Henderson-Hasselbalch equation, describing the pH-dependent equilibrium between ammonium and ammonia, or nitrous acid and nitrite as follows:

$$\text{mM ammonia} = \text{mM (ammonium plus ammonia)} - \frac{\text{mM (ammonium plus ammonia)}}{\left(\frac{10^{(-\text{pH})}}{10^{(-\text{pK}_a)}} + 1 \right)}$$

where mM (ammonium plus ammonia) = mM ammonium plus ammonia in bioassays or soil; $\text{pK}_a = 9.25$ (24°C).

$$\text{mM nitrous acid} = \frac{\text{mM (nitrous acid plus nitrite)}}{\left(\frac{10^{(-\text{pH})}}{10^{(-\text{pK}_a)}} + 1 \right)}$$

where mM (nitrous acid plus nitrite) = mM nitrous acid plus nitrite in bioassays or soil; $\text{pK}_a = 3.33$ (24°C).

Statistical analyses. The concentration of ammonia that reduced the percent germination of microsclerotia to 5% (effective concentration, EC_{95}) in bioassays was determined by probit analysis. The computer software Probit Program (61) was used to fit data sets to the probit distribution, to estimate EC_{95} values and confidence intervals ($P = 0.05$). Analysis was conducted with the

mean value of the six replicates of a treatment. Data sets appropriately fitted the probit distribution as determined by the chi-square test ($P < 0.05$).

A treatment in soil microcosms resulting in germination of microsclerotia of 5% or less was considered effective in killing the pathogen. This threshold value was chosen because a germination of 5% or less for microsclerotia retrieved from field plots amended with meat and bone meal (37 t/ha), soy meal (37 t/ha), and poultry manure (66 t/ha), and soil microcosms containing the amended field soil, corresponded to a negligible incidence of Verticillium wilt of field-grown potatoes (14,31).

For the soil microcosm experiments, values for percent germination of microsclerotia were angular transformed (arcsine square root) to normalize the variance prior to data analysis (34). For the last date of analysis of an experiment, the transformed data were analyzed by analysis of variance using the program Systat for Macintosh (release 5.2.1, SPSS Inc., Chicago, IL) for the effects experimental repeat, treatment, and their interaction. There was no significant effect of experimental repeat or its interaction with treatment ($P > 0.05$). Thus, the germination of microsclerotia and the chemical analyses for each analysis date and treatment were averaged ($n = 6$) for the two repeats of each experiment, and the results are presented in figures.

RESULTS

Toxicity of ammonia to microsclerotia. Germination of *V. dahliae* microsclerotia was not affected by the pH of the solid me-

Fig. 1. Effect of ammonia and nitrous acid on the germination of *Verticillium dahliae* microsclerotia. **A**, Photograph of representative agar plates containing medium set to various levels of pH and concentrations of ammonium chloride. The germination of microsclerotia is evident by the formation of colonies. **B**, Germination as a function of the concentration of ammonia in agar medium in **A**. **C**, Germination in response to pH and concentration of nitrite in solution. **D**, Germination as a function of the concentration of nitrous acid in the solutions in **C**. Data shown are an average ($n = 6$, ± 1 SE) of the results from two experiments with three replicates each.

dium at 0 mM ammonium or the concentration of ammonium at pH 7 (Fig. 1A). The germination was reduced when the levels of pH and ammonium increased. The germination of microsclerotia decreased at concentrations of ammonia greater than 0.6 mM and was completely inhibited at concentrations greater than 5 mM (Fig. 1B). An EC_{95} value of 4.4 mM ammonia (confidence interval = 3.3 to 8.9; $P = 0.05$) was determined with this bioassay. The microsclerotia that failed to germinate after 2 weeks were transferred to regular soil pectate tergitol agar. These microsclerotia did not germinate and were thus considered dead. The pH of all agar plates was within 0.5 units of that set at the start (limit of resolution of test strips), indicating that the concentration of ammonia in the medium was not drastically altered during the incubation.

Fig. 2. Effect of duration of exposure of *Verticillium dahliae* microsclerotia to solutions of **A**, ammonia and **B**, nitrous acid. Microsclerotia were placed in solutions for 8 h, 1 and 4 days, or for 4 days above solutions (4 day gas). Data shown are an average ($n = 6$, ± 1 SE) of the results from two experiments with three replicates each.

TABLE 2. Concentration of nitric oxide (NO), nitrogen dioxide (NO₂), and nitrous oxide (N₂O) in headspace of jars (250 ml) containing solutions of nitrous acid (50 ml)^a

Nitrous acid (mM)	Concentration in headspace above solutions		
	NO (μmol/liter)	NO ₂ (μmol/liter)	N ₂ O (μmol/liter)
0	0.05 (<0.01)	0.08 (<0.01)	0.04 (<0.01)
0.029	0.96 (0.05)	0.17 (0.01)	0.04 (<0.01)
0.117	2.36 (0.17)	0.48 (0.01)	0.11 (0.03)

^a Jars were sealed and incubated at 24°C in the dark for 1 day. Values are the average ($n = 6$) of two experiments with three replicates each. Standard error of the mean shown in parenthesis.

In the absence of nitrite, the germination of microsclerotia was not affected by the pH of the buffer solution (Fig. 1C). The germination was reduced by increasing acidity of solutions and concentrations of nitrite (Fig. 1C). The germination of microsclerotia decreased at concentrations of nitrous acid greater than 0.01 mM and was completely prevented at concentrations greater than 0.2 mM (Fig. 1D). An EC_{95} value of 0.154 mM nitrous acid (confidence interval = 0.133 to 0.186; $P = 0.05$) was determined for this 1-day exposure.

Fig. 3. Effect of addition of meat and bone meal to site B soil on germination of *Verticillium dahliae* microsclerotia retrieved from soil or from the headspace above soil, the soil pH, and the concentrations (mM) of ammonia and nitrous acid in soil solution. Meat and bone meal was added at concentrations of 0, 0.5, 1, 2, and 2.5% of oven-dry soil with or without the addition of the nitrification inhibitor, dicyandiamide (360 mg/kg of soil). Data shown are the average ($n = 6$, ± 1 SE) of the results from experiments with three replicates each.

Effect of duration of exposure. The germination of microsclerotia decreased with increasing concentration and duration of exposure to ammonia (Fig. 2A) or nitrous acid (Fig. 2B) in solution. After exposure for 4 days, the germination of microsclerotia was completely inhibited at 10 mM ammonia (Fig. 2A). At this duration of exposure, the EC_{95} value was 4.5 mM (4 to 5.5 mM; $P = 0.05$) ammonia. After exposure for 4 days, germination was completely inhibited at 0.03 mM nitrous acid (Fig. 2B) and the EC_{95} value was 0.02 mM (0.018 to 0.024 mM; $P = 0.05$).

Fig. 4. Effect of addition of ammonium sulfate to site M soil on germination of *Verticillium dahliae* microsclerotia, the soil pH, and the concentrations (mM) of nitrous acid, nitrite, and nitrate in soil solution. Ammonium sulfate was added at concentrations of 0, 200, and 400 mg of nitrogen/kg of oven-dry soil with or without the addition of the nitrification inhibitor, dicyandiamide. Data shown are the average ($n = 6$, ± 1 SE) of the results from two experiments with three replicates each.

The pH of the solutions and the concentrations of ammonia and nitrous acid after 4 days were similar to those at the start of the experiment. Sodium chloride added to solutions at the same concentrations as ammonium chloride or sodium nitrite did not affect the germination of microsclerotia (data not shown).

Effect of exposure in atmosphere. Ammonia was also lethal as a gas volatilized from solution ($EC_{95} = 3.4$ mM NH_3 in solution, 3.0 to 4.1 mM; $P = 0.05$) (Fig. 2A). Similarly, germination was prevented for microsclerotia suspended for 4 days above the 0.120 mM solution of nitrous acid (Fig. 2B). Of the three nitrogen oxide gases measured, nitric oxide was present in the highest concentration in the headspace of the jars above the solutions of nitrous acid (Table 2).

Toxicity of ammonia and nitrous acid in site B soil. Germination of microsclerotia retrieved from soil (Fig. 3A) or headspace (Fig. 3B) of the 2.5% treatment was prevented in the presence of very high levels of ammonia at day 10 (60 mM) (Fig. 3D). The germination of microsclerotia was reduced to near 0% by day 7 in the 2% treatment. However, as ammonia dissipated, germination increased to above 20% by the end of the study. The 0.5 and 1% meat and bone meal treatments prevented the germination of microsclerotia retrieved from soil and the headspace by the end of the study. In these treatments, the soil pH decreased from a maximum on day 4 (Fig. 3C) as the products of nitrification, nitrite, nitrate (data not shown), and nitrous acid accumulated (Fig. 3E). The addition of dicyandiamide with the 2 and 2.5% treatments prevented the accumulation of nitrite and nitrate, and a reduction in the soil pH (Fig. 3H). It also promoted an earlier death of microsclerotia retrieved from soil (Fig. 3F) and headspace (Fig. 3G), and the accumulation of ammonia (Fig. 3I). Treatment with dicyandiamide prevented the accumulation of nitrous acid (Fig. 3J) and promoted the survival of microsclerotia in the 0.5 and 1% treatments retrieved from soil (Fig. 3F) and the headspace (Fig. 3G).

Inhibition of nitrification in ammonium sulfate-amended soil. Germination of microsclerotia declined to zero 2 weeks after addition of ammonium sulfate as the soil proceeded to acidify and accumulate the products of nitrification, nitrite, nitrate, and nitrous acid (Fig. 4). The addition of dicyandiamide prevented the loss in germination of microsclerotia and the accumulation of nitrous acid (Fig. 4).

Toxicity of ammonia in site L soil. Germination was prevented when the concentration of meat and bone meal was increased to 4% of the soil mass (Fig. 5A). At 4% treatment, an increase in soil pH (Fig. 5B) and the accumulations of ammonium (Fig. 5C) and ammonia to a maximum of 63 mM (Fig. 5D) occurred. With the 3% treatment, the germination of microsclerotia was reduced to 30% after 7 days of incubation and coincided with a modest accumulation of ammonia (to 18 mM). However, the reduction in germination was temporary and it returned to that of the control treatment by day 28 as ammonia dissipated. With the 2 and 3% treatments, the soil pH decreased below that of the control by day 14. This coincided with a reduction in the concentration of ammonia (Fig. 5D) and an increase in nitrite (Fig. 5E) and nitrate (Fig. 5F).

The germination of microsclerotia declined to less than 5% in 1 week, with the highest concentration of urea (0.36%) in site L soil (Fig. 6A). This coincided with an increase in the soil pH (Fig. 6B) and ammonium (Fig. 6C), and the accumulation of ammonia to 30 mM (Fig. 6D). At 1 week, low levels of ammonia (<15 mM) accumulated with the 0.27 and 0.18% treatments, reducing germination to 15 and 30%, respectively. However, the germination increased in these treatments to 50% by day 28 as ammonia dissipated in soil. A rapid reduction in the soil pH with the lowest rate of urea addition occurred as nitrate accumulated (Fig. 6F). Substantial concentrations of nitrite accumulated in the three highest treatments attaining approximately 100 mM by day 21 (Fig. 6E).

DISCUSSION

Despite numerous reports that nitrogenous organic amendments suppress soilborne plant pathogens and pests, none have demonstrated that it is possible to reduce application concentrations to practical levels (3,28). Understanding how nitrogenous organic amendments kill pathogens may contribute to the lowering of concentrations. The generation of ammonia from amendments has been most often the mechanism implicated in killing pathogens (12,21,39,60). Nitrous acid has also been implicated in killing pathogens (35,39,60). The results presented here demonstrate conclusively that ammonia and nitrous acid killed a pathogen in soil receiving organic and synthetic nitrogen amendments.

Ammonia and nitrous acid rather than their ionized counterparts, ammonium and nitrite, respectively, were lethal to microsclerotia of *V. dahliae* in bioassay and soil studies. This agrees with the findings of others that ammonia (16,36,39,46,60) and nitrous acid (10,39,41,47,60,66) are toxic to several pathogens and pests.

The lethal concentration of nitrous acid was 2 orders of magnitude lower than for ammonia. Furthermore, half the concentration of the organic nitrogenous amendment, meat and bone meal, was added to produce nitrous acid than ammonia toxicity. How either compound is toxic, let alone, why nitrous acid is more toxic, is not known. Concentrations of ammonia in the loam soil between 5 and 20 mM reduced the germination of microsclerotia to less than 50%. However, when concentrations of ammonia dissipated, the germination of microsclerotia increased. This suggests that sublethal levels of ammonia are fungistatic.

Unlike their ionized counterparts, ammonia and likely nitrous acid readily penetrate the outer membrane of cells (8,37). Ammonia is thought to kill cells by disrupting membranes (50), eliminating proton gradients across membranes (17), assimilation of ammonia into glutamine depleting citrate and malate (51), and the exhaus-

tion of the chemical energy of cells removing cytosolic ammonia across a concentration gradient (6). In comparison, nitrous acid is a strong oxidizing agent capable of rapidly destroying vital compounds in organisms including glycosaminoglycans, porphyrins, free amino groups, and ascorbic acid (1,4,13,64). Nitrous acid reacts with secondary amines yielding nitrosamines that are carcinogenic (29). Nitrous acid is a potent mutagen and has been used to generate mutant lines of viruses, bacteria, fungi, and plants (7,15,53,65). The mutagenic effect of nitrous acid to *Saccharomyces cerevisiae* has been characterized and shown to be restricted on the S-phase of cell growth (15), resulting in the increased frequency of cross-linking of DNA (15,44).

Ammonia evolved from solution or soil was presumably toxic to microsclerotia. Likewise, ammonia volatilized from soil was toxic to other pathogens (9,10,26). Unlike ammonia, nitrous acid is not a volatile. It is unclear how microsclerotia suspended above solutions of nitrous acid and soil were killed. Nitric oxide, and to a lesser extent, nitrogen dioxide gas, were present in the headspace above solutions of nitrous acid. These gases are formed from the self-decomposition of nitrous acid (5) or reactions with reduced metals and organic compounds such as amides (62). Nitric oxide is produced in soil receiving nitrogenous amendments such as fertilizers or cattle slurry (20). Thus, the death of microsclerotia in the headspace of solutions and soil may have occurred because of the dissolution of nitric oxide (and possibly nitrogen dioxide) to form nitrous acid in the water film surrounding the pathogen. Nitric oxide is directly toxic to mammalian cells (42). Thus, it cannot be ruled out that nitric oxide and nitrogen dioxide may contribute to directly killing *V. dahliae* microsclerotia.

Within 1 week after the incorporation of meat and bone meal or urea to soil, ammonia accumulated in soil proportional to the concentration of amendment. The germination of microsclerotia was reduced at concentrations of ammonia greater than approximately

Fig. 5. Effect of addition of meat and bone meal to site L soil on A, germination of *Verticillium dahliae* microsclerotia; B, soil pH; and concentrations (mM) of C, ammonium; D, ammonia; E, nitrite; and F, nitrate in soil solution. Meat and bone meal was added at concentrations of 0, 2, 3, and 4% of oven-dry soil. Data shown are the average ($n = 6$, ± 1 SE) of two experiments with three replicates each.

Fig. 6. Effect of addition of urea to site L soil on A, germination of *Verticillium dahliae* microscerotia; B, soil pH; and concentrations (mM) of C, ammonium; D, ammonia; E, nitrite; and F, nitrate in soil solution. Urea was added at concentrations of 0, 0.09, 0.18, 0.27, and 0.36% of oven-dry soil. Data shown are the average ($n = 6$, ± 1 SE) of the results from two experiments with three replicates each.

5 mM and was completely prevented at concentrations greater than approximately 35 mM. When such levels (>35 mM) were attained, they persisted for the remaining 3 weeks of each study. The accumulation of nitrate was negligible, indicating that the persistence of ammonia was due to the inhibition of nitrification. Nitrifying bacteria are intolerant of high levels of ammonium, ammonia, and pH (19), such as those found in the soils receiving the highest rates of the amendments. The products of nitrification, nitrite and nitrate, accumulated at concentrations of ammonia of less than approximately 35 mM. This implies that nitrification proceeded, resulting in the acidification of the soil and the reduction in concentrations of ammonia.

The accumulation of nitrous acid in the sandy soils examined resulted from nitrification. Nitrification resulted in the production of nitrite and acidification of soil producing nitrous acid. The addition of the nitrification inhibitor, dicyandiamide, prevented the accumulation of nitrous acid in meat and bone meal and ammonium sulfate-treated soils and negated the ability of the amendments to kill microscerotia. The nitrification inhibitor, nitrapyrin, promoted the survival of *Fusarium oxysporum* f. sp. *dianthi* (35) and *Ralstonia solanacearum* (39) in urea and urea plus CaO-treated soil, respectively. Together, with the findings presented here, there is considerable evidence of nitrous acid killing pathogens in amended soil.

The amount of an organic nitrogenous amendment required to generate ammonia toxicity to a pathogen was dependent upon the soil examined. The concentration of meat and bone meal in site B soil (2.5%) was lower than in site L soil (4%) to generate similar levels of ammonia lethal to microscerotia. Because pH is critical to the generation of ammonia, it is reasonable that ammonia-generating materials such as anhydrous ammonia, urea, oil seed meals, and urea plus CaO were effective in killing pathogens in alkaline but not acidic soils (39,54). It was unexpected that site L

soil with an initial pH of 8.1 did not accumulate more ammonia than site B soil of pH 6. Furthermore, nitrous acid did not accumulate in site L soil. Clearly, soil characteristics other than initial pH control the accumulation of ammonia and nitrous acid. Determination of these characteristics is crucial to predicting and improving the efficacy of nitrogenous amendments in the field. Several soil characteristics that likely limit ammonia accumulation were higher in site L soil and include cation exchange capacity (63), nitrification rate (45), organic carbon (38), and clay content (38). The relation of these characteristics in controlling the accumulation of ammonia is under investigation.

Lower concentrations of organic and synthetic nitrogenous amendments were required to generate nitrous acid than ammonia toxicity. It is thus surprising that nitrous acid, as a means to control pathogens, has been overshadowed by ammonia. This likely discrepancy is because nitrous acid occurs in low concentration. A laboratory must be equipped to measure very low concentrations of nitrite (0.25 to 1 mg of N per liter) in water suspensions of soil in conjunction with the determination of the soil pH.

Having determined when and to what concentrations ammonia and nitrous acid accumulate in soil from an organic nitrogenous amendment and synthetic fertilizer, it is now possible to explore reducing addition concentrations to practical levels. Particularly, exploiting a nitrous acid toxicity mechanism in acid soil is a means to reduce amendment concentrations. Furthermore, identifying soil characteristics that control the accumulation of either toxin allows for use of the lowest amendment concentration to kill pathogens.

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