Relation of Ammonia and Nitrous Acid to Suppression of *Phytophthora* in Soils Amended with Nitrogenous Organic Substances

Peter H. Tsao and J. J. Oster

Professor and staff research associate, respectively, Department of Plant Pathology, University of California, Riverside 92521.
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ABSTRACT

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Amendment of natural soils with chicken manure and urea at 2 and 0.1%, respectively, caused soil pH to rise from near neutral to above 8.6 and then fall to the acid range. Soil nitrogen analyses confirmed correspondence of this pH pattern to the processes of ammonification and nitrification. Results of in vitro tests with NH_4OH and KNO_2 solutions demonstrated that *Phytophthora* propagule germination was inhibited by low concentrations of NH_3 and HNO_2. A minimum of 4 ppm NH_3 or 0.3 ppm HNO_2 reduced *Phytophthora cinnamomi* sporangium germination to $\leq$ 1%; 6 ppm NH_3 or 0.5 ppm HNO_2 reduced *P. parasitica* sporangium germination to $\leq$1%; 17 ppm NH_3 or 0.9 ppm HNO_2 reduced *P. parasitica* chlamydospore germination to less than 10 and 5%, respectively. Additional in vitro tests with ammonium and nitrite salts showed that toxicity resided in the NH_4^+ or NO_2^- portion of a compound. Furthermore, the pH dependency of germination inhibition in the ammonium/ammonia and nitrite/nitrous acid solutions demonstrated that NH_3 and HNO_2, respectively, are primarily responsible for the inhibition. NH_4OH at a given concentration was more toxic at pH 8 than at pH 6, but KNO_2 was more toxic at pH 6 than at pH 8. Sufficiently high concentrations of NH_4^+ and NO_2^- were detected in amended soils at soil pH values favoring their presence in the nonionized forms, NH_3 and HNO_2, respectively, to account for significant germination inhibition. Therefore, these toxicants are responsible, at least in part, for *Phytophthora* suppression in amended soils.

Additional key words: organic amendment, suppressive soil.

The form of amendment nitrogen applied to the soil influences plant disease development (21). Chicken manure, alfalfa meal, and certain other nitrogenous organic amendments are known to reduce activity and/or survival of *Phytophthora cinnamomi* and *P. parasitica* in soil (7,14,43,44,48). There are also reports on the inhibitory effects of urea soil amendment on soilborne pathogens as diverse as *Fusarium* (38), *Phytophthora* (26,45), *Poria* (30), and *Sclerotium* (17).

A rise and a fall in soil pH, corresponding to ammonification and nitrification (2,28), commonly have been observed after soil amendment with low C:N ratio organic compounds (1,13,33,43,45). Work on avocado root rot has shown ammonium/ammonia and nitrite/nitrous acid are toxic to *P. cinnamomi* (13,45,49). Gilpatrick (13) reported that soils amended with 5% alfalfa meal accumulated 60–197 ppm ammonia within 7 days after amendment, but contained no more than did unamended soil (2–8 ppm) by 28–58 days. *P. cinnamomi* was not recoverable from infected avocado roots incubated for 3–4 days in a soil amended 1 wk previously. Furthermore, 17 ppm NH_3 in phosphate buffer at pH 7 was toxic to mycelium and zoospores of *P. cinnamomi* (13). Ammonia toxicity also has been implicated as a possible mechanism of soil fungistasis in certain natural soils (23,34) and in chitin-amended soil (36), and inhibition of spore germination by volatile inhibitor(s) is enhanced in soils with alkaline pH (20,23,34). Henis and Chet (16,17) reported the death of *Sclerotium rolfsii* sclerotia in soil amended with ammonia and ammonium-containing compounds. Smiley et al (40) demonstrated the fungitoxicity of anhydrous ammonia applied to soil and of nitrification-produced nitrite to *Fusarium* spp. Zentmyer and Bingham (49) reported that low concentrations of nitrite were toxic to *P. cinnamomi* mycelium and zoospores in vitro and prevented *Phytophthora* root rot of avocado in liquid culture.

Ammonium/ammonia and nitrite/nitrous acid each exist in

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equilibrium in aqueous solutions. The proportion of the ionized to the nonionized form depends on the solution pH and their concentrations can be calculated by the Henderson-Hasselbalch equation (47). The nonionized form of each compound penetrates cell membranes more easily than do the ionized forms and it is usually more toxic (12,22,29,46). The ionized form also may play a role in toxicity by changing membrane permeability (12,35).

The present paper reports the inhibitory effects of ammonium/ammonia and nitrite/nitrous acid, at several pH values, on propagules of *P. cinnamomi* and *P. parasitica,* and their relationship to *Phytophthora* inhibition in soils amended with nitrogenous organic substances. Some of the results have been briefly reported (32).

MATERIALS AND METHODS

Test fungi. Two species of *Phytophthora* were used in these studies: *P. cinnamomi* Rands, isolate T139 (= Pc 40), pathogenic to avocado, and *P. parasitica* Dast. (*P. nicotianae* Breda de Haan sensu Waterhouse), isolate T131, pathogenic to citrus.

Soils. Two Fallbrook sandy loam soils were collected from avocado groves, screened to pass a 2-mm sieve, and briefly air dried. Soil pH was 6.9–7.1 in a 1:1 (w/v) soil:water mixture and 6.0–6.5 in a 1:1 (w/v) soil:0.01 M $CaCl_2$ mixture (39).

Sporangium production on nylon mesh squares. A modification of the methods of Chen and Zentmyer (8) and Schoulties and Baker (37) was used. Small (1-mm square) blocks of cornmeal agar (Difco, 17 g/L) containing mycelium were cut from the edge of a young *Phytophthora* culture and placed in 15 ml of cleared V8–$CaCO_3$ broth (19) in 90-mm-diameter petri dishes. For *P. cinnamomi,* small colonies (12–15 mm diameter) of actively growing mycelium were transferred, after 24 hr of dark incubation at 25 C, to 15 ml of 1/5-strength V8–$CaCO_3$ broth and grown for another 24 hr. *P. parasitica* mycelium was incubated in the dark continuously for 48 hr. After being washed three times (20 ml per wash) in sterile double-deionized water (hereafter called water) to remove nutrients, *P. cinnamomi* mycelial mats were transferred to another clean 90-mm-diameter petri dish, containing 20 ml of a mineral salts solution (8). The mats were washed at hourly intervals for 4 hr with 20 ml of salts solution at each wash and placed in 15 ml of salts solution under continuous light (two General Electric [Louisville, KY 40225] Cool-White 40-W fluorescent lamps, 54 cm from the culture, at an intensity of 1,700 $\mu W/cm^2$, luminosity of 2,400 lux [220 ft-c]) for 24 hr at 25 ± 1 C. *P. parasitica* mats were washed briefly three times with water and incubated in the dark in 5 ml of water per 90-mm diameter petri dish at 25 C for 24 hr. Zoospore differentiation and release were induced by washing sporangium-bearing mycelial mats three times with water and incubating them at 18 C in 15 ml of water for 30–60 min for *P. cinnamomi* and 15–20 min for *P.* parasitica. Concentrations of the zoospore suspensions were adjusted to 2,000/ml for *P. cinnamomi* and 500/ml for *P. parasitica,* and combined with V8–$CaCO_3$ broth to give 1/5-strength and 4/5-strength V8–$CaCO_3$ for *P. cinnamomi* and *P. parasitica,* respectively. To establish single-zoospore colonies (37) 5 ml of the resulting suspensions were pipetted into 90-mm-diameter petri dishes and incubated at 25 C for 16–20 hr, when small colonies had become visible. Squares of nylon mesh (12 × 12 mm size, 100-μm opening, monofilament nylon cloth [Nitex HC 100]; Tetko, Inc., Elmsford, NY 10523) were autoclaved, rinsed in water, drained of liquid, and arranged in 150-mm-diameter polystyrene dishes, 20 squares per dish. Each square was covered with 0.1 ml of 1/5-strength (for *P. cinnamomi*) or full-strength (for *P. parasitica*) V8–$CaCO_3$ broth and a single-zoospore colony was inserted with a glass needle into the mesh at the center of the square. The dishes were then incubated at 25 C in the dark for 4–6 hr, colony position was examined and re-centered if necessary, and an additional 0.1 ml of V8–$CaCO_3$ broth was added to each square. Incubation was continued for 18 more hours until the mycelium covered each square. The squares were then washed three times with water (50 ml per wash) to remove nutrients and transferred to a separate, dry, clean, polystyrene petri dish. Squares with *P. cinnamomi* mycelium again were washed in salts solution (at hourly intervals for 4 hr, 50 ml per wash), individually arranged and covered with 0.3 ml of salts solution, and incubated under light for 24 hr at 25 ± 1 C. Squares with *P. parasitica* mycelium were washed briefly with water (instead of prolonged washing with salts solution) but covered also with 0.3 ml of salts solution (to prevent premature zoospore release) and incubated in the dark for 24 hr at 25 C. Mature sporangia formed abundantly within 24 hr.

Chlamydospore production and harvest. Chlamydospores of *P. parasitica* were produced by submerging mycelium in water and incubating at 18 C as described previously (42). For chlamydospore harvest, a modification of an earlier method (42) was used. The chlamydospore-bearing mycelial mats (with inoculum plug removed) were pooled from the bottles, washed by repeatedly immersing mats in cheesecloth bags in successive changes of water, and blended for 3 min in 150 ml of water in a Sorvall Omnimixer (control setting of 8) (Sorvall, Inc., Norwalk, CT 06856). Blending was done with the cup submerged in an icewater bath to prevent overheating. The mycelial fragments were partially separated from the chlamydospores in the resulting suspension by a short (15 sec), low-speed centrifugation in a clinical centrifuge (400 *g* at the end of 15 sec). The supernatant was replaced with water and the pellet was resuspended. After repeating the 15-sec centrifugation once, the resulting suspension was then centrifuged for 1 min at 1,500 *g*, and each of the several capped centrifuge tubes was inverted repeatedly to resuspend the upper, white, predominantly mycelial layer from the lower, light brown chlamydospore pellet. The supernatant was decanted and the pellet resuspended in water. The process was repeated once more or until the white layer was removed. Pellets were then washed by resuspending and centrifuging (3 min at 1,500 *g*) three times in three changes of water, again suspended in 10 ml of water, and an aliquot was removed for counting on a Hawksley eelworm counter (Gelman Hawksley Ltd., Lancing, Sussex, England). The chlamydospore suspension was diluted with water to a concentration of 20,000 spores per milliliter. Viability was tested by incubating spores in 60 μg/ml rose bengal (18,27). Viability (or percent unstained chlamydospores) was generally between 90–100%.

Preparation of test solutions and buffers. Aqueous solutions of NH_4OH, $(NH_4)_2SO_4$, NH_4Cl, KNO_2, $NaNO_2$, glucose, asparagine, and Good's buffers (15) each were prepared at four times (or eight times for glucose and asparagine) the final test concentrations. Good's buffers used were MES, MOPS, and TAPS (range of final concentrations 0.01–0.03 M, depending on toxicant concentrations) and were adjusted to pH 5, 6, 7, or 8 with 1 M KOH. Solutions were then sterilized by filtration through a 0.22-μm pore size Millipore filter. Glucose and asparagine solutions were then mixed 1:1 (v/v) to give four times the test concentration of the combined glucose-asparagine solution. Concentrations of solutions of all chemicals were calculated on a w/v basis and are reported herein in molarity (M) or ppm (mg/L).

Sporangium germination tests. Each nylon mesh square supporting a sporangium-bearing mat was briefly rinsed in water and immediately placed in a 60-mm-diameter glass petri dish containing 2 ml of control or test solution. To prevent premature zoospore release, squares were transferred quickly, and no sporangium production dish was allowed to remain open longer than 10 min. Each experiment contained water control (2 ml water per dish), buffer control (0.5 ml buffer, 1.5 ml water), and various toxicant solutions (0.5 ml toxicant, 0.5 ml buffer, and 1 ml water) with three replicates for each treatment. Solution pH was taken before and after incubation. Petri dishes were incubated at 25 ± 1 C for 30 min to allow the toxicants to act on the sporangia (containing undifferentiated protoplasm) and then at 18 C for 90 min to induce zoospore formation and release. Squares were immediately mounted in lactophenol on microscope slides under coverslips, and sealed. One hundred sporangia representative of the entire mycelial mat were read for indirect germination (zoospore release) and grouped into five categories. They were: 1 = empty; 2 = partially full, but without a papilla, and containing a number of zoospores; 3 = full with intact papilla, but with differentiated zoospores; 4 = full, but with undifferentiated protoplasm; and 5 = full, or partially full, but with abnormal protoplasm. The first two categories were

combined and considered as germinated sporangia to obtain percent germination. Averages of three replicates per treatment were obtained and the germination is reported as percent of buffer control.

Chlamydospore germination tests. Methods used were similar to those described by Holdaway and Tsao (18). Freshly washed chlamydospore suspensions (20,000 spores per milliliter) were pipetted in 0.5-ml aliquots into each 60-mm-diameter glass petri dish containing control or test solution to reach a final volume of 2 ml. Each experiment contained a water control (0.5 ml of spore suspension, 1.5 ml of water per dish), buffered glucose-asparagine control (0.5 ml of spore suspension, 0.5 ml glucose-asparagine solution, 0.5 ml of buffer, and 0.5 ml of water), and various toxicant solutions (0.5 ml of spore suspension, 0.5 ml of glucose-asparagine, 0.5 ml of buffer, and 0.5 ml of toxicant). The final spore concentration was 5,000/ml. All treatments were in triplicate. Dishes were incubated in 20 × 150-mm circular moist chambers for 16–18 hr in the dark at 25 C. Solution pH was measured before and after incubation. Germination was terminated by placing dishes at 1 C before reading. A spore was considered germinated if the germ tube length exceeded its width. One hundred spores from each replicate were counted, and percent germination was obtained from averages of three replicates per treatment and reported as percentage of the buffered glucose-asparagine control.

Soil amendment. Soils were thoroughly mixed with 0.1% (w/w, dry weight basis) powdered urea or 2% ground, dried, processed chicken manure (Pro Mac) (McAnally Enterprises, Inc., Yucaipa, CA 92399). Each 250-ml polystyrene container received 110 g (dry weight) of unamended or amended soil, was provided with a perforated (2-mm pore) lid to allow aeration, and enclosed in a polyethylene bag to maintain moisture (45). All treatments were in triplicate and incubation was at 24–25 C and 14–23% moisture (matric potential of about −0.05 to −0.15 bar).

Soil pH determination. A 5-g (dry weight equivalent) soil sample was taken from each replicate at 0, 1, 2, 3, 4, 7, 10, 14, 21, and 28 days, stirred with 5 ml of water, incubated 0.5 hr, stirred again, and the pH was measured.

Soil nitrogen analyses. Nitrogen determinations for NH_4^+-N, NO_2^--N, and NO_3^--N (5) were performed at 0, 4, 7, 11, 14, and 21 days. Each 25-g soil sample was shaken with 250 ml of 2 N KCl for 1 hr. The resulting extract was steam-distilled with MgO and the distillate was titrated with 0.005 N H_2SO_4 to determine NH_4^+-N. Addition of Devarda alloy to the undistilled portion followed by a second distillation and titration of the resulting distillate with H_2SO_4 was performed to determine NO_2^--N + NO_3^--N. The NO_3^--N was determined by first distilling a duplicate sample to eliminate NH_4^+-N, then treating the undistilled portion with sulfamic acid, and finally distilling with Devarda alloy followed by titration of the resulting distillate with H_2SO_4. The NO_2^--N was determined by subtraction of NO_3^--N from NO_3^--N + NO_2^--N. Results were obtained as ppm N (μg N per g soil) and then converted to ppm of respective ions.

Calculations with the Henderson-Hasselbalch equation. The Henderson-Hasselbalch equation (47) was applied to the ammonium/ammonia and nitrite/nitrous acid equilibria by substituting specific values in the generalized equation, pH = pKa + log [conjugate base] / [conjugate acid]. Since concentrations of NH_4^+ and NO_2^- and their respective pKa values were known in these studies (from concentration of NH_4OH or KNO_2 in test solutions or NH_4^+ and NO_2^- in soil nitrogen analyses), these and the measured pH values were substituted in the appropriate equation, $pH = 9.25 + \log [NH_3] / [NH_4^+]$ − $[NH_3]$ or $pH = 3.37 + \log [NO_2^-] - [HNO_2] / [HNO_2]$. The equation was then solved for either NH_3 or HNO_2 concentration.

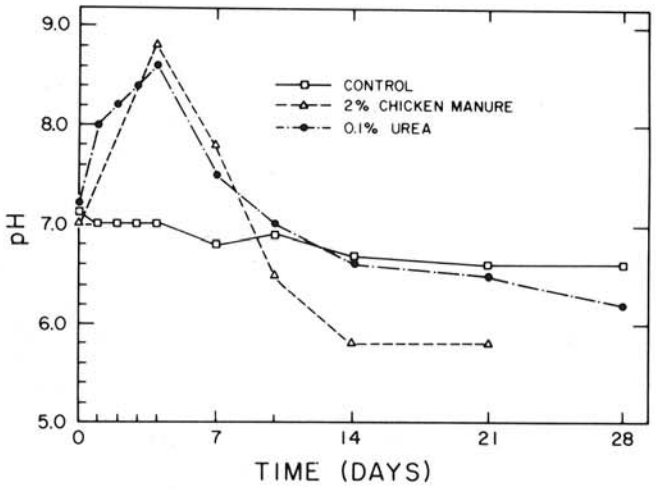

Fig. 1. Changes in pH in unamended control soils (average of four experiments) and in soils amended with 2% chicken manure (average of two experiments) or 0.1% urea (average of four experiments) after amendment day (day 0).

RESULTS

Changes in pH in amended soils. Soil pH after amendment with 2% chicken manure or 0.1% urea rose rapidly from near neutrality on day 0 to above pH 8.6 on day 4, and declined rapidly to the acid range, below that of the unamended control by day 14 and afterwards (Fig. 1). The pH of control soil declined slowly from pH 7.1 on day 0 to pH 6.6 by day 28.

Toxicity of ammonia to *P. cinnamomi* sporangia. Ammonium hydroxide at four concentrations (0.001, 0.002, 0.004, and 0.006 M) was tested at each of three pH levels (pH 6, 7, and 8) for toxicity to sporangia of *P. cinnamomi*. Buffers were uninhibitory to sporangium germination at the test concentrations. Solution pH did not vary more than 0.2 pH units during incubation. Two

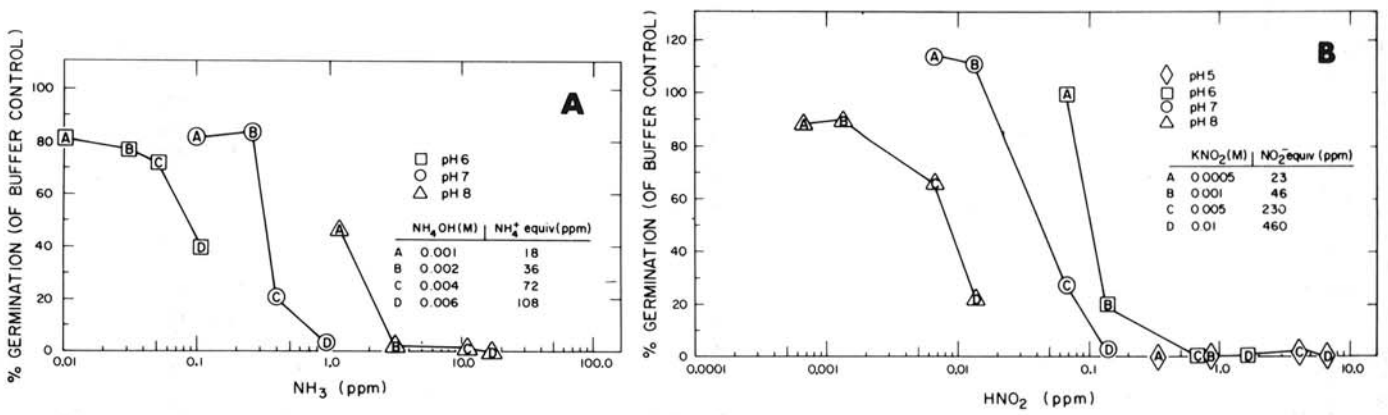

Fig. 2. Percent germination of *Phytophthora cinnamomi* sporangia in **A)** NH_4OH solutions at about pH 6, 7, and 8, and **B)** KNO_2 solutions at about pH 5, 6, 7, and 8. Data are plotted as percent germination (expressed as percentage of appropriate buffer control) vs equilibrium concentrations (ppm) of the toxic form, NH_3 in **A** or HNO_2 in **B**. Compare these equilibrium concentrations of the nonionized forms, NH_3 and HNO_2, with the equivalent concentrations (M) of NH_4OH and KNO_2 and the nonequilibrium concentrations (ppm) of the ionized forms, NH_4^+ and NO_2^-, respectively.

experiments were conducted; the results of one experiment are summarized in Fig. 2A. At any given pH, sporangium germination decreased with increasing NH_4OH concentration. For example, at pH 7 germination was 82% in 0.001 M NH_4OH but only 4% in 0.006 M NH_4OH. With pH increase, germination generally decreased at a given NH_4OH concentration. For example, germination in 0.002 M NH_4OH was 77% at about pH 6 (0.03 ppm NH_3), but was only 1% at about pH 8 (3 ppm NH_3) (Fig. 2A).

Averaged percent germination figures (from one to six experiments) were similar to those presented in Fig. 2A. However, at many of the concentrations, the range in germination among experiments was wide (range varying from 0.4 to 70.7%, average 27.5%). In a few cases, this may be due to variation in pre-incubation solution pH (0.2 pH unit at pH 6 and 7, 0.4 pH unit at pH 8) of repeat experiments which produced a range in NH_3 concentrations (range varying from 0.006 to 7 ppm) at a given NH_4OH concentration. However, germination within an experiment always declined with increasing NH_4OH concentration and increasing pH, and always was 1% or less when NH_3 concentration exceeded 4 ppm.

Toxicity of nitrous acid to *P. cinnamomi* sporangia. Potassium nitrite at four concentrations (0.0005, 0.001, 0.005, and 0.01 M) was tested at each of four pH levels (pH 5, 6, 7, and 8) for toxicity to sporangia of *P. cinnamomi*. Buffers were uninhibitory and, as with NH_4OH tests, solution pH was constant within 0.2 pH units during incubation. Two experiments were conducted; Fig. 2B summarizes the results of one experiment. As with NH_4OH, sporangium germination decreased with increasing KNO_2 concentration at any given pH. For example, at pH 7 germination in 0.0005 M KNO_2 was 114%, but it was only 1% in 0.01 M KNO_2. In contrast with that in NH_4OH, germination in KNO_2 decreased with decreasing pH at a given KNO_2 concentration. For example, germination in 0.005 M KNO_2 was 66% at about pH 8 (0.007 ppm HNO_2) but was 0% at about pH 6 (0.7 ppm HNO_2) (Fig. 2B).

Averaged percent germination figures (one to six experiments) were similar to those presented in Fig. 2B. Again, the range in germination was wide (range varying from 0 to 81.7%, average 30.7%), and may in a few cases be due to variation in pre-incubation solution pH (0.2 pH unit at pH 5, 6, and 7, and 0.1 pH unit at pH 8) which produced a range in HNO_2 concentrations (range varying from 0.003 to 0.6 ppm) at a given KNO_2 concentration. However, germination within an experiment always declined with increasing KNO_2 concentration and decreasing pH, and was always 1% or less when HNO_2 concentration exceeded 0.3 ppm.

Effects of various ammonium and nitrite salts on *P. cinnamomi* sporangium germination. Several compounds of ammonium (NH_4OH, $[NH_4]_2SO_4$, and NH_4Cl, all at 0.002 M NH_4^+ equivalent) and nitrite (KNO_2 and $NaNO_2$ at 0.005 M) were tested, in one experiment, for toxicity to *P. cinnamomi* sporangia at about pH 6 and 8 (Table 1). At a given pH, there was no significant difference ($P = 0.05$, according to Duncan's multiple range test) in sporangium germination among the ammonium compounds. Similarly, there was no significant difference in germination between KNO_2 and $NaNO_2$ solutions at about pH 6, but $NaNO_2$ allowed significantly higher germination than did KNO_2 in solutions at about pH 8. However, at that pH, germination was high in both cases (Table 1). These results showed convincingly that inhibition of sporangium germination was due to the NH_4^+ or NO_2^- portions of the compounds. Repetition of the pH-dependent toxicity pattern observed in previous experiments (Fig. 2A and B) further indicated that the nonionized NH_3 or HNO_2 formed in equilibrium with NH_4^+ or NO_2^-, respectively, in the solutions are the primary inhibitory compounds.

Relative sensitivity of propagules of *P. cinnamomi* and *P. parasitica* to NH_3 and HNO_2. Sporangia of the two *Phytophthora* species and/or chlamydospores of *P. parasitica* were used in five experiments to test the relative sensitivity of these propagules to the two toxicants at several concentrations (Tables 2 and 3).

Table 2 summarizes the data of germination experiments with NH_4OH solutions tested at about pH 8. *P. cinnamomi* sporangia were generally more sensitive than *P. parasitica* sporangia which were, in turn, more sensitive than *P. parasitica* chlamydospores (eg, 2.3, 26.9, and 97% germination in 0.002 M NH_4OH, respectively). The range of percent germination was wide at the NH_4OH concentrations tested in different experiments, however, and sporangia of *P. cinnamomi* did not always exhibit lower germination than those of *P. parasitica* in all tests. In a single experiment (Table 4), germination of *P. cinnamomi* and *P. parasitica* sporangia was tested simultaneously in NH_4OH solutions at 0.001, 0.002, 0.004, and 0.01 M, at both pH 6 and 8. At pH 6, germination of *P. cinnamomi* sporangia was significantly ($P = 0.05$) less than that of *P. parasitica* at all concentrations (Table 4). At pH 8, germination of sporangia of both fungi was zero or near zero at all concentrations.

The data of germination experiments with KNO_2 solutions tested at about pH 6 are summarized in Table 3. Again, as with NH_4OH, *P. cinnamomi* sporangia generally were more sensitive than *P. parasitica* sporangia which were, in turn, more sensitive than *P. parasitica* chlamydospores (eg, 33.4, 59.9, and 87.2% germination in 0.001 M KNO_2, respectively). Again, as with NH_4OH experiments, the range of percent germination was wide at the KNO_2 concentrations tested in different experiments, and sporangia of *P. cinnamomi* did not always exhibit lower germination than those of *P. parasitica* in all tests. Germination of sporangia of the two fungi was tested simultaneously in KNO_2 solutions at 0.0005, 0.001, 0.002, and 0.005 M, at both pH 6 and 8 (Table 4). At pH 6, germination of *P. cinnamomi* sporangia was significantly ($P = 0.05$) less than that of *P. parasitica* at 0.001 and 0.002 M KNO_2. There were no significant differences in germination at an uninhibitory concentration (0.0005 M KNO_2) and at a completely

TABLE 1. Germination of *Phytophthora cinnamomi* sporangia in solutions of NH_4^+ and NO_2^- compounds at about pH 6 (5.7–5.9) and 8 (7.9–8.2). Compare the equilibrium concentrations of the nonionized forms, NH_3 and HNO_2, with the equivalent concentrations (M) of NH_4OH and KNO_2 and the nonequilibrium concentrations (ppm) of the ionized forms, NH_4^+ and NO_2^-, respectively

Compound	Concentration			Approximate pH of test solutions	Effective toxicant concentration (ppm) at equilibrium		Germination[a] (%)
	M	ppm equivalent NH_4^+	ppm equivalent NO_2^-		NH_3	HNO_2	
NH_4OH	0.002	36		6	0.01		95.3 A[b]
$(NH_4)_2SO_4$	0.001	36		6	0.002		95.3 A
NH_4OH	0.002	36		8	2.6		6.8 C
$(NH_4)_2SO_4$	0.001	36		8	1.5		9.5 C
NH_4Cl	0.002	36		8	1.5		10.1 C
KNO_2	0.005		230	6		0.9	0.7 C
$NaNO_2$	0.005		230	6		0.9	1.5 C
KNO_2	0.005		230	8		0.01	73.0 B
$NaNO_2$	0.005		230	8		0.01	94.6 A

[a] Expressed as percentage of appropriate buffer control. Germination of sporangia was terminated after incubation in 2 ml of toxicant or control solutions in 60-mm-diameter petri dishes at 25 C for 30 min and then at 18 C for 90 min.

[b] Figures with same letter are not significantly different ($P = 0.05$, according to Duncan's multiple range test).

inhibitory concentration (0.005 M KNO_2). At pH 8, germination of *P. cinnamomi* sporangia was significantly less than that of *P. parasitica* at 0.002 and 0.005 M KNO_2. There were no significant differences in germination at uninhibitory concentrations (0.0005 and 0.001 M KNO_2).

Concentrations of NH_3 and HNO_2 in amended soils. Based on soil pH values obtained at the time of nitrogen analysis, estimates of equilibrium concentrations of ammonium/ammonia and nitrite/nitrous acid were obtained by application of the Henderson-Hasselbalch equation. Soil pH changes were similar to those in Fig. 1. There were no detectable changes in concentrations of NH_3 (about 0.04 ppm) or HNO_2 (about 0.005 ppm) in the unamended soil throughout the 21 days. Concentrations of NH_3 in 0.1% urea-amended soil reached a maximum of 93 ppm (in equilibrium with 281 ppm NH_4^+) by day 4, but declined to 1.4 ppm (in equilibrium with 254 ppm NH_4^+) by day 7 and to 0.1 ppm (in equilibrium with 112 ppm NH_4^+) by day 21. Concentrations of NH_3 in 2% chicken manure-amended soil reached a maximum of 183 ppm (in equilibrium with 550 ppm NH_4^+) by day 4, and declined to 41 ppm (in equilibrium with 576 ppm NH_4^+) by day 7 and to 0.4 ppm (in equilibrium with 252 ppm NH_4^+) by day 21. For the purpose of comparison with in vitro toxicity, germination of *P. cinnamomi* sporangia was reduced to ≤1% by 4 or more ppm NH_3; that of *P. parasitica* sporangia to ≤1% by 6 or more ppm NH_3; and that of *P. parasitica* chlamydospores to less than 10% by 17 or more ppm NH_3.

Concentrations of HNO_2 in 0.1% urea-amended soil reached a maximum of 0.3 ppm (in equilibrium with 239 ppm NO_2^-) by day 21 (the last day of soil analysis readings). Concentrations of HNO_2 in 2% chicken manure-amended soil also reached a maximum of 0.3 ppm (in equilibrium with 258 ppm NO_2^-) by day 21. For the purpose of comparison with in vitro toxicity, germination of *P. cinnamomi* sporangia was reduced to ≤1% by 0.3 or more ppm HNO_2; that of *P. parasitica* sporangia to ≤1% by 0.5 or more ppm HNO_2; and that of *P. parasitica* chlamydospores to less than 5% by 0.9 or more ppm HNO_2.

DISCUSSION

Results of previous work have shown that soil amendment with chicken manure, urea, or certain other nitrogenous organic amendments at appropriate concentrations inhibited sporangium

TABLE 2. Relative sensitivity of sporangia of *Phytophthora cinnamomi* (P.c.) and sporangia and chlamydospores of *P. parasitica* (P.p.) to NH_3, in NH_4OH solutions tested at about pH 8(7.9–8.4). Results are averages of one to five experiments

Propagule	Concentration NH_4^+ M	Concentration NH_4^+ ppm equivalent	Concentration NH_3 ppm effective (equilibrium)	Germination[a] (%)
P.c. sporangia	0.001	18	1	21
	0.002	36	3	2.3
	0.004	72	8	0.1
P.p. sporangia	0.001	18	1	35.5
	0.002	36	3	26.9
	0.004	72	5	2.1
P.p. chlamydospores	0.001	18	1	109
	0.002	36	2[b]	97[b]
	0.004	72	4[b]	96.6[b]

[a] Expressed as percentage of appropriate buffer control. Germination of sporangia was terminated after incubation in 2 ml of toxicant or control solutions in 60-mm-diameter petri dishes at 25 C for 30 min and then at 18 C for 90 min. Germination of chlamydospores was terminated after incubation at 25 C for 16–18 hr.

[b] Results of one experiment. All other data are averages of two to five experiments.

TABLE 3. Relative sensitivity of sporangia of *Phytophthora cinnamomi* (P.c.) and sporangia and chlamydospores of *P. parasitica* (P.p.) to HNO_2, in KNO_2 solutions tested at about pH 6 (5.9–6.2). Results are averages of one to five experiments

Propagule	Concentration NO_2^- M	Concentration NO_2^- ppm equiv.	Concentration HNO_2 ppm effective (equilibrium)	Germination[a] (%)
P.c. sporangia	0.0005	23	0.05	84.6
	0.001	46	0.1	33.4
	0.005	230	0.6	0.2
P.p. sporangia	0.0005	23	0.05	98.2
	0.001	46	0.1	59.9
	0.005	230	0.5	1.3
P.p. chlamydospores	0.0005	23	0.04[b]	102[b]
	0.001	46	0.1	87.2
	0.005	230	0.5	24.8

[a] Expressed as percentage of appropriate buffer control. Germination of sporangia was terminated after incubation in 2 ml of toxicant or control solutions in 60-mm diameter petri dishes at 25 C for 30 min and then at 18 C for 90 min. Germination of chlamydospores was terminated after incubation at 25 C for 16–18 hr.

[b] Results of one experiment. All other data are averages of two to five experiments.

TABLE 4. Simultaneous comparison of percent germination of *Phytophthora cinnamomi* (P.c.) and *P. parasitica* (P.p.) sporangia in NH_4OH or KNO_2 solutions at about pH 6 (6.2–6.3) and 8 (8.0–8.4). Results are given as percent germination at the concentration (ppm) of the toxic form (NH_3 or HNO_2) at equilibrium. Compare with equivalent nonequilibrium concentrations of NH_4OH and KNO_2 (M) or NH_4^+ and NO_2^- (ppm)

Approximate pH of test solutions	Concentration NH_4^+ M	Concentration NH_4^+ ppm equiv.	Concentration NH_3 ppm effective (equilibrium)	Germination[a] (%) P.c.	Germination[a] (%) P.p.	Concentration NO_2^- M	Concentration NO_2^- ppm equiv.	Concentration HNO_2 ppm effective (equilibrium)	Germination[a] (%) P.c.	Germination[a] (%) P.p.
6	0.001	18	0.01	84.6 B[b]	104.1 A	0.0005	23	0.03	106 A[b]	100 AB
6	0.002	36	0.03	47.1 C	72.5 B	0.001	46	0.07	87.1 BC	108.3 A
6	0.004	72	0.06	12.1 D	38.7 C	0.002	92	0.14	1.8 E	57.3 D
6	0.01	180	0.16	0 D	0 D	0.005	230	0.34	0 E	2.7 E
8	0.001	18	1.5	0 D	2.3 D	0.0005	23	0.0004	101.8 A	109.2 A
8	0.002	36	4	0 D	0 D	0.001	46	0.001	106.6 A	105.3 A
8	0.004	72	6	0 D	0.7 D	0.002	92	0.002	51.5 D	101.4 A
8	0.01	180	12	0.6 D	0.7 D	0.005	230	0.005	8.1 E	79.4 C

[a] Expressed as percentage of appropriate buffer control. Germination of sporangia was terminated after incubation in 2 ml of toxicant or control solutions in 60-mm-diameter petri dishes at 25 C for 30 min and then at 18 C for 90 min.

[b] Figures with same letter are not significantly different ($P = 0.05$, according to Duncan's multiple range test).

germination of *Phytophthora* and eliminated or greatly reduced *Phytophthora* propagules in the soil (13,14,26,43–45). Present studies showed that, after soil amendment, an initial rapid rise in pH occurred (accompanying ammonification) followed by a pH decline (accompanying nitrification), as well as the detection of ammoniacal, nitrite, and nitrate nitrogen predominantly at periods when soil pH was high and low, respectively. These results confirmed a mechanism for *Phytophthora* suppression in amended soil suggested earlier by Gilpatrick (13) and Zentmyer and Bingham (49). Our in vitro tests demonstrated that ammonium/ammonia and nitrite/nitrous acid are highly toxic to *Phytophthora* propagules and, therefore, at least partially responsible for *Phytophthora* inhibition in amended soils. Furthermore, the data suggested that nonionized forms, NH_3 and HNO_2, of these toxicants were primarily responsible for the effect. Maximum toxicant quantities at concentrations greatly inhibitory in vitro were formed in amended soils when pH favored their presence in toxic nonionic forms. These toxicants also may adversely affect fungal survival either singly or additively as first ammonification and then nitrification occur in amended soils. It is likely that this same mechanism might also be partly responsible for suppression of other pathogens in soils amended with nitrogenous organic substances (26,30,36,38).

Most of these organic amendment substances, when added to soil, cause an increase of general as well as antagonistic soil microflora (43). Therefore, pathogen suppression by soil amendment can be the result of microbial antagonism, which has indeed been the most commonly postulated mechanism for disease or pathogen control in amended soils (3,10,17,30,31,43,48). *Phytophthora* suppression in soils amended with relatively high concentrations of chicken manure, urea, or other nitrogenous organic substances is drastic, is often complete within 2 wk or less (43–45), and is therefore less likely the result of microbial antagonism. Increased antagonistic microbial population in amended soils conceivably can play a role in long-term *Phytophthora* suppression, however.

During ammonification-nitrification in 0.1% urea and 2% chicken manure amended soils, quantities of ammonium/ammonia formed were far in excess of concentrations inhibitory to *Phytophthora* propagules in vitro, while nitrite/nitrous acid also were present in quantities sufficient to significantly reduce *Phytophthora* propagule germination. Furthermore, pH changes in amended soils were such that highest amounts of ammonium/ammonia and nitrite/nitrous acid were present when pH favored predominance of the more toxic, nonionized forms, NH_3 and HNO_2 (at high and low pH, respectively). Use of the Henderson-Hasselbalch equation to calculate concentrations of NH_3 and HNO_2 at the appropriate soil pH gave an estimate of these nonionized toxicant concentrations in the soil. Toxicant presence mostly in films of moisture around soil or toxicant-emanating amendment particles may have resulted in a local soil pH different from bulk soil pH and thus produced even higher nonionized toxicant concentrations. Volatilization of NH_3 and toxicant interactions with minerals and soil microbes (other than ammonification-nitrification) may, on the other hand, decrease aqueous toxicant concentrations (6,23,24,33). Uneven amendment distribution in the soil and possibly abatement of toxicant action by soil nutrients also may lower toxicant effectiveness.

In vitro germination experiments with *P. cinnamomi* sporangia in solutions containing ammonium/ammonia and nitrite/nitrous acid confirmed the nonionized forms, NH_3 and HNO_2, are the principal toxic compounds responsible for the inhibition. It is well known that NH_3 and HNO_2 are inhibitory to various microorganisms, and ammonium- or nitrite-containing fungitoxic or bacteritoxic compounds have been used to preserve fruit, vegetable, meat, and other food products (4,11,22,25). Mechanisms of microbial inhibition by NH_3 and HNO_2 are not well known, but are perhaps similar to those known in plants and animals (22,29). Toxicity of the ionized forms, NH_4^+ and NO_2^-, to *Phytophthora* cannot be ruled out, however, since plotting of percent germination against calculated concentrations of NH_3 or HNO_2 at a given pH did not always yield the expected straight line. In addition, although certain different NH_4OH or KNO_2 concentrations at each of several pH levels produce equivalent amounts of nonionized toxicants in solution, the amount of ionized compound present in equilibrium with the nonionized form is different for each different NH_4OH or KNO_2 concentration. Effects of these ions on membrane permeability or interaction of buffer with toxicant may account for these discrepancies. Solution pH itself does not account for this effect; high percent germination (usually greater than 90%) occurred in buffer controls of all pH levels tested (pH 6, 7, and 8).

P. parasitica chlamydospores were much less sensitive than its sporangia, which were usually less sensitive than sporangia of *P. cinnamomi,* to these toxicants. However, it should be realized that indirect germination of *Phytophthora* sporangia was tested in water, while germination of *Phytophthora* chlamydospores was tested in nutrient solution, because the former is a nutrient independent process and the latter is nutrient dependent (27,41). It is possible, therefore, that presence of glucose and asparagine in the chlamydospore germination medium could partially nullify toxicant effects on germination (9).

It is not yet known whether the inorganic toxicants at concentrations tested in vitro were fungistatic or fungicidal after prolonged incubation. Preliminary data suggest that treatments were fungistatic for sporangia and chlamydospores after 2 and 18 hr of incubation, respectively. Results of germination inhibition tests of chlamydospores on polycarbonate membranes buried in chicken manure-amended soils showed them to be fungicidal by 9 or 14 days, but only fungistatic by 1 or 3 days (J. La Favre and P. H. Tsao, *unpublished*). Additional work on the mode of action of organic nitrogenous soil amendment is in progress.

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