LABORATORY AND FIELD STUDIES ON THE EFFECT OF AMMONIA ON THE PROPAGULES OF *PYTHIUM ULTIMUM*, *MACROPHOMINA PHASEOLINA*, *THIELAVIOPSIS BASICOLA*, AND *HELMINTHOSPORIUM* SPP.

by

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ABSTRACT

LABORATORY AND FIELD STUDIES ON THE EFFECT OF AMMONIA ON THE PROPAGULES OF *PYTHIUM ULTIMUM*, *MACROPHOMINA PHASEOLINA*, *THIELAVIOPSIS BASICOLA*, AND *HELMINTHOSPORIUM* SPP.

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Soybean meal was incorporated into a field of sandy loam soil artificially infested with *Pythium ultimum* Trow. Initially, the populations of this pathogen increased then decreased to about the levels found in unamended soil.

Sandy loam soil was artificially infested with *P. ultimum*, *Thielaviopsis basicola*, and *Macrophomina phaseolina* (Tassi) Goid. [=*M. phaseoli* (Maubl.) Ashby]. Soil was then placed into clay tiles sunk into a field and treated with urea. Ammonia, generated from hydrolysis of urea, increased with the amount of urea applied. Population densities were significantly, and often markedly, decreased by 1.0, 0.5, and 0.25% urea (based on soil dry weight); 0.1% urea appeared to be effective at high soil temperatures, but was ineffective at low temperatures.

Soil assays for P. ultimum and M. phaseolina were improved. The assay for P. ultimum makes use of rapid propagule germination and growth rate of this fungus. Soil dilutions were placed in 2.5 mm diam. wells in 2% water agar in 90 x 15 mm petri dishes. Hyphae from germinated sporangia growing from the cylindrical walls of the wells were counted. M. phaseolina sclerotia were recovered from soil with an efficiency of 95-97% by flotation using 60% sucrose. Viable surface-sterilized sclerotia were enumerated by culturing on a selective medium.

The effect of ammonia on viability of ^{14}C-labeled propagules of Cochliobolus victoriae, Cochliobolus sativus, and M. phaseolina and exudation from the propagules was studied in a model fungistatic system with nutrient-independent or nutrient-dependent propagules or in soil amended with urea from which ammonia was generated. In soil, ammonia stimulated exudation of ^{14}C-labeled compounds, but their metabolism by the soil microbiota was suppressed. Short-term exposures of a few hours in a model fungistatic system with high free ammonia concentrations (up to 10,000 mg/L) stimulated a burst of exudation which was usually associated with death of the propagules. Lower concentrations increased exudation less, but exudation tended to be maintained at a level greater than that in nontreated controls. Viability was reduced when the propagules were continuously exposed to long-term doses which were nonlethal for shorter durations. In a fixed-volume system, which imposed low nutrient stress, ammonia (10-100 mg/L) had a sparing effect on the increased exudation brought on by high pH alone; as ammonia concentration was increased (100-1000 mg/L), the sparing effect was lost

and exudation increased, accompanied by decreased viability. Therefore, high pH by itself increased exudation, but it did not account for the greater exudation effected by ammonia, nor for its lethal effects.

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Chapter 1

GENERAL INTRODUCTION

A large body of research has been dedicated to the use of crop residues and soil amendments in regard to soil-borne plant pathogens. The goal has been to provide some biological or cultural control of these fungi (Baker and Cook, 1974). Explanations of how soil amendments function to reduce the population densities of root-infecting fungi have taken different approaches. Competition between soil microorganisms (Garrett, 1970; Lindsey, 1965; Maurer and Baker, 1965; Snyder et al., 1959) has been one explanation given. Soil microbes generally reside in a quiescent or dormant state for most of their life cycles due to low available carbon (Lockwood, 1977). Sustained growth and reproduction may occur with the introduction of organic amendments. When the amendment is rich in carbon and low in nitrogen, microorganisms stimulated to grow may utilize the soil nitrogen in the surrounding environment as well as from decomposing organic amendments. Snyder et al. (1959) found that Fusarium solani f. sp. phaseoli was less active as a pathogen under such competitive conditions, but when the C:N ratio was lowered, either from a high nitrogen content organic amendment or by the addition of a nitrogen source, the pathogen became active and caused severe disease (Maurer and Baker, 1965). Lindsey (1965) has demonstrated competition in the presence of limiting

nitrogen between Fusarium solani f. sp. phaseoli and Fusarium roseum f. sp. cerealis.

Stimulatory and suppressive substances resulting from amendment decomposition affecting resistant fungal structures under conditions of fungistasis (Balis and Kouyeas, 1979; Lockwood, 1977; 1981) have been used to explain the effects of soil amendments. Where an organic amendment was involved, stimulatory compounds were believed to function largely as energy sources rather than as nonnutritive activators (Lockwood, 1981). For instance, the addition of oat straw, corn stover, or alfalfa hay to soil infested with Thielaviopsis basicola resulted in reduced populations of the pathogen due to stimulation of germination of endoconidia and chlamydospores with subsequent germling lysis occurring before formation of secondary endoconidia and chlamydospores (Lewis and Papavizas, 1975; Sneh et al., 1976). In addition, microbial activity may increase with a concomitant rise in the level of soil fungistasis, following introduction of organic amendments to soil. Volatile (Balis, 1976; Balis and Kouyeas, 1979; Pavlica et al., 1978; Schippers and Palm, 1973) and nonvolatile (Lockwood and Filonow, 1981) compounds have been described, which may play a role in suppressing germination and growth of fungi. However, little emphasis has been placed on these suppressive substances to control soil-borne disease.

Investigations suggested that organic soil amendments with high C:N ratios were more effective than organic amendments with low C:N ratios in reducing disease severity or populations densities of Fusarium (Lewis and Papavizas, 1975; Maurer and Baker, 1965; Snyder et al.,

1959). Zakaria and Lockwood (1980) reported the first use of oilseed meals as soil amendments to reduce Fusarium populations. They compared 10 plant or animal residues as amendments to reduce populations of Fusarium in soil. In these experiments, the plant residues with higher C:N ratios were ineffective, whereas crab shell and oilseed meals reduced population densities to 0.001 or less of the original within 4-6 weeks at concentrations as low as 0.25% (w/w) in the laboratory. Of the three oilseed meals tested (linseed, cottonseed, and soybean), soybean meal reduced the population the most in a moisture range of -3.0 to 0 bars matric potential. Attempts to carry these results from closed containers in the laboratory to the field failed. However, subsequent work by Zakaria et al. (1980) demonstrated a greater effectiveness of the oilseed meals in closed containers than in open containers. When soil containing high Fusarium populations was placed in planchets and incubated on the surface of oilseed meal-amended soils in closed containers, population densities decreased, which strongly implicated one or more volatile inhibitors. Ammonia was consistently detected in trapping solutions from oilseed meal-supplemented soils, appearing as soon as 2 days after amendment with soybean meal and 3 days with linseed and cottonseed meal. Gilpatrick (1969a,1969b) suggested that ammonia produced from the decomposition of plant tissues in soil was the active volatile principle responsible for reduction of some root diseases. Ammonia has been detected in alkaline soils (Ko et al., 1974a; 1974b; Pavlica et al., 1978), in soils made alkaline by the addition of lime (Hora and Baker, 1974; Pavlica et al., 1978), and in soils to which chitin was added (Shippers and Palm, 1973). Zakaria et al. (1980) suggested that the mechanism of propagule reduction by ammo-

nia-yielding amendments was not germination followed by lysis (Lewis and Papavizas, 1975; Sneh et al., 1976), but direct killing by ammonia (Rush and Lyda, 1982a; 1982b; Schippers and Palm, 1973; Tsao and Zentmyer, 1979). This interpretation agrees with the speculation of Lewis and Papavizas (1975) that ammonia was the toxic volatile decomposition product from immature plant tissues and corn tissues with low C:N ratios, which adversely affected the survival of Rhizoctonia soloni in soil.

The objective of this research was to continue the research begun by Zakaria et al. (1980) on the use of soybean meal as a soil amendment to control soil-borne fungal pathogens. An initial objective was to ascertain whether soybean meal amendment would effectively reduce populations of pathogenic fungi other than Fusarium. In the course of this research, urea was found to be a more practical alternative in terms of availability, handling, and cost, and moreover would not act as a C-source. Direct and indirect effects of urea on propagules of P. ultimum, T. basicola, and M. phaseolina were studied in the laboratory and field. Since ammonia is the likely active component derived from the decomposition of soybean meal or urea in soil, its effect on various propagules was studied in the laboratory. In particular, the interactive effect of ammonia and pH on viability and exudation from fungal propagules was examined.

Chapter 2

EFFECT OF SOYBEAN MEAL AMENDMENT OF SOIL ON POPULATIONS OF *PYTHIUM ULTIMUM* AND *THIELAVIOPSIS BASICOLA* IN THE LABORATORY

2.1 INTRODUCTION

Zakaria et al. (1980) demonstrated that volatile degradation products of various oilseed meal amendments decreased *Fusarium* populations in soil and that the principle volatile involved was ammonia. As a continuation to this work, similar studies were done to determine whether *Pythium ultimum* and *Thielaviopsis basicola* populations were similarly affected.

2.2 MATERIALS AND METHODS

2.2.1 Fungi and preparation of inocula.

Pythium ultimum was isolated from soybean seedling pieces (Schlub and Lockwood, 1981). *Thielaviopsis basicola* isolate 157 was isolated from diseased soybeans in Michigan. *P. ultimum* was maintained on carrot agar and *T. basicola* on potato-dextrose-yeast agar (PDYA). Carrot agar was prepared by autoclaving 30 g of sliced carrots in 250

ml of distilled H_2O and decanting the supernatant solution into 20 g of agar in 750 ml of distilled H_2O. PDYA was made by adding 5 g of Difco yeast extract per L of PDA.

Pythium ultimum was grown on hemp seed broth (Schlub and Schmitthenner, 1978) in glass containers for 10 or more days at 24-26°C. Mycelial mats were collected, rinsed with sterile distilled water, and homogenized in an Omni-mixer (Ivan Sorvall, Norwalk, CT 06856) for 1 min at 10,400 rpm. The homogenate was passed through an 80 µm nylon mesh filter to retain the mycelial fragments. The sporangia in the filtrate were collected on 15 or 20 µm nylon mesh screens. The collected sporangia were rinsed and resuspended in water for use.

Thielaviopsis basicola was cultured on PDY broth at 24-26°C for 1 1/2 months. Mycelial mats were collected, rinsed with water, and homogenized in an Omni-mixer for 10 min at 7,400 rpm. The homogenate was repeatedly centrifuged and resuspended in water to remove nutrients. The suspension was rehomogenized for 10 minutes, passed through a 38 µm nylon screen, repeatedly centrifuged and resuspended in water, decanting off as many endoconidia as possible during each cycle. Finally the chlamydospores and remaining endoconidia were collected on paper filters and air-dried to inactivate remaining endoconidia. As needed, chlamydospores were resuspended in water with the help of a tissue grinder. This spore suspension was used to infest soil.

2.2.2 Soybean degradation volatiles affecting Pythium ultimum.

Simple chambers were constructed from large plastic petri dishes (147 x 15 mm). A rubber septum was installed in the cover to permit the introduction and removal of material from an inner well consisting of a small plastic petri dish (57 x 15 cm) bottom. Initially the inner well was filled with 1.0 ml of distilled water as a trap for ammonia. Fifty g of sandy loam soil, made up to -0.1 bars matric potential, was placed in the chambers around the inner well. The soil was amended with 1% (w/w) of either alfalfa or an oilseed meal amendment--cottonseed, linseed, or soybean seed meal. The amendments were ground in a Wiley mill to pass through a 20-mesh screen (0.87 mm). Nonamended soil was used as controls. The chambers were sealed with Parafilm (American Can Company, Greenwich, CT 06830) to reduce evaporation and gas exchange.

The soil in the chambers was incubated for 3 days at 24-26°C after which 1.0 ml of water containing approximately 10,000 P. ultimum sporangia was introduced into the inner well. The soil was incubated an additional day before germinability of the sporangia was determined. Germinability was determined by adding 0.2 ml nutrient solution to the inner wells to stimulate germination of viable sporangia. The soil was incubated for 18 h and then the percentage of germination was determined directly. The amount of ammonia generated was determined by removal of samples from the inner well and analyzing them by gas chromatography. A Varian Aerograph 1400 series gas chromatograph (Varian Instruments, Palo Alto, CA 94303) equipped with a

glass column (1.83 m x 0.64 mm x 2 mm i.d.) packed with Chromosorb 103 (Johns-Manville, Denver, CO 80217) was used to identify and quantify ammonia. The oven temperature used was 100°C with an injector port temperature of 190°C and a flame ionization detector temperature of 280°C. The carrier gas was He at a flow rate of 25 cc/min. Concentrations of ammonia as low as 10 ng could be detected.

2.2.3 Effect of soybean meal in soil on Pythium ultimum and Thielaviopsis basicola.

The effect of soybean meal amendment in soil artificially infested with P. ultimum was examined. Sandy loam soil was artificially infested with sporangia and equilibrated for several days at 24-26°C. The soil was brought up to -0.1 bars matric potential and amended with 1.0% (w/w) soybean meal. The soil was kept in sealed 11 x 18 cm pint size plastic bags in styrofoam cups. Population determinations were made initially and 2, 4, 6, 8 and 10 days after the soil was amended, using the method of Stanghellini and Hancock (1970). Disease reduction in the same soils was also examined. Amended and unamended soils were air-dried for about a month after the experiment was terminated, then were diluted 1:1 (v/v) with uninfested soils. Thirty-five soybean seeds (variety 'Hark') were planted in amended soil, unamended soil, and in uninfested soil. Ten days later, emergence was determined.

Similiar experiments were done with open containers and with different concentrations of soybean meal amendment incubated for an extended period.

The effect of soybean meal amendment on T. basicola population in soil was studied. Sandy loam soil was infested with chlamydospores of T. basicola and incubated at least a week for the population to stabilize. The soil was brought up to -0.1 bars matric potential and amended with 1.0% (w/w) soybean meal. The soil was kept in sealed 11 x 18 cm pint size plastic bags in styrofoam cups. Population counts were taken at 0, 2, 5, 8, and 12 days. To assay populations of T. basicola, soil samples were suspended in 0.2% water agar and shaken for 1 h before additional dilutions were made in 0.2% water agar. TBM-C medium was poured into plates containing soil dilutions to estimate populations of T. basicola (Maduewesi et al., 1976). A similiar experiment was done with open containers.

2.3 RESULTS

2.3.1 Soybean degradation volatiles affecting Pythium ultimum.

Soil amended with soybean meal generated the largest amount of ammonia followed by linseed and cottonseed meal- amended soils in closed chambers (Table 1). Ammonia was not detected in the control or alfalfa-amended soils. Germinability of the sporangia in separate containers within the chambers was inversely correlated with the concentration of ammonia. These results agree with those of Zakaria et al. (1980). Alfalfa meal reduced germination of the sporangia, but its effect was small compared to the effect of the oilseed meals.

TABLE 1. EFFECT OF AMENDMENT OF SOIL WITH ORGANIC MATERIALS (1%, w/w) ON VIABILITY OF SPORANGIA OF PYTHIUM ULTIMUM AND ON THE GENERATION OF AMMONIA

Amendment (meal)	Germinability of sporangia (%)	Ammonia (ppm)[x]
Control	92	0
Alfalfa	70	0
Cottonseed	24	432
Linseed	18	767
Soybean	12	1006

[x]Ammonia concentration in the trap solutions were determined by gas chromatography

2.3.2 Effect of soybean meal in soil on Pythium ultimum.

In a preliminary experiment, P. ultimum populations in soil dropped from an initial population of 1175 to 20 and 0 CFU/g 4 and 8 days, respectively, after amendment with 1% soybean meal. In contrast the population in unamended soil dropped to 675 and 525 CFU/g after 4 and 8

days. When longer time periods were examined in another experiment, the population in soil amended with soybean meal decreased to a much lower level than that in unamended soil (Table 2). The odor of ammonia was apparent when the plastic bags were opened to remove samples for population determinations.

Disease severity was greatly reduced by the soybean amendment. Ten days after planting soybean seeds, 71% of the seeds emerged from the amended soil, 60% from the uninfested soil, and only 14% from the unamended soil.

TABLE 2. *PYTHIUM ULTIMUM* POPULATIONS IN SOIL AT DIFFERENT TIME PERIODS AFTER AMENDMENT WITH SOYBEAN MEAL, IN CLOSED CONTAINERS

	CFU/g ± s.e.	
Days	Unamended	Amended (1%)
0	1456 ± 316	1456 ± 316
2	1370 ± 212	1200 ± 625
4	1640 ± 351	1446 ± 750
6	980 ± 116	80 ± 37
8	680 ± 166	10 ± 6
10	280 ± 86	2 ± 2

When the experiment was done without sealing the plastic bags, the population of P. ultimum increased rapidly in the amended soil, then declined steeply and rapidly (Table 3). The most rapid population decrease occurred from the 2nd to the 6th day after amendment.

TABLE 3. PYTHIUM ULTIMUM POPULATIONS IN SOIL AT DIFFERENT TIME PERIODS AFTER AMENDMENT WITH SOYBEAN MEAL, IN OPEN CONTAINERS

	CFU/g ± s.e.	
Days	Unamended	Amended (1%)
0	546 ± 62	546 ± 62
2	134 ± 27	14,720 ± 1,966
4	270 ± 48	1032 ± 573
6	338 ± 50	12 ± 6
10	378 ± 95	24 ± 16

The experiment with the open containers was done with different concentrations of soybean meal and for longer incubation intervals. The population increased rapidly following amendment (Table 4). This was followed by a drop in the population which was more rapid at the higher amendment concentrations. With the 1.0 and 0.5% concentrations, the population dropped below the level of detection by the 10th and 25th day, respectively. With the 0.25% amendment, the population remained higher than the initial levels over a longer time period. Only after 47 days had the population dropped substantially below the control. The experiment was repeated and the same trends observed.

TABLE 4. *PYTHIUM ULTIMUM* POPULATIONS IN SOIL AT DIFFERENT TIME PERIODS AFTER AMENDMENT WITH SOYBEAN MEAL, IN OPEN CONTAINERS

	CFU/g ± s.e.			
	Percent soybean meal (w/w)			
Days	0	0.25	0.5	1.0
0	428 ± 14	428 ± 14	428 ± 14	428 ± 14
2	378 ± 33	5250 ± 144	3950 ± 758	7675 ± 4130
4	315 ± 34	5675 ± 359	5525 ± 335	45 ± 30
7	218 ± 31	4175 ± 1020	4500 ± 641	8 ± 8
10	142 ± 59	3825 ± 1076	4350 ± 478	0
16	238 ± 34	3975 ± 2286	38 ± 16	---
25	160 ± 36	2555 ± 1450	0	---
47	188 ± 30	74 ± 478	0	---

2.3.3 Effect of soybean meal in soil on *Thielaviopsis basicola*.

The *T. basicola* population remained relatively constant in the unamended soil in the closed containers (Table 5). However, population

reduction was observed as soon as 2 days after the amendment was applied. By the 8th day, T. basicola was barely detectable.

TABLE 5. THIELAVIOPSIS BASICOLA POPULATIONS IN SOIL AT DIFFERENT TIME PERIODS AFTER AMENDMENT WITH SOYBEAN MEAL, IN CLOSED CONTAINERS

	CFU/g ± s.e.	
Days	Unamended	Amended (1%)
0	11,400 ± 510	11,400 ± 510
2	11,000 ± 2,098	5,000 ± 1,826
5	15,800 ± 1,393	1,000 ± 632
8	10,580 ± 1,814	8 ± 6
12	9,440 ± 435	0

In open containers, the T. basicola population reduction was delayed until after the fourth day (Table 6). By 8 days, the population dropped to zero or near zero. Again the control population remained relatively stable throughout the first 10 days of the experiment, then decreased by about 35%. The odor of ammonia was apparent when either the sealed and unsealed plastic bags were sampled.

TABLE 6. *THIELAVIOPSIS BASICOLA* POPULATIONS IN SOIL AT DIFFERENT TIME PERIODS AFTER AMENDMENT WITH SOYBEAN MEAL, IN OPEN CONTAINERS

	CFU/g ± s.e.	
Days	Unamended	Amended (1%)
0	10,520 ± 1,424	10,520 ± 1,424
2	10,300 ± 1,241	10,500 ± 2,363
4	11,240 ± 1,423	11,433 ± 7,201
6	11,012 ± 2,498	3,802 ± 1,984
8	11,800 ± 1,281	0
10	11,940 ± 1,546	0
12	7,740 ± 406	0
14	7,475 ± 547	0

2.4 DISCUSSION

The relative production of ammonia from soybean, linseed, cottonseed, and alfalfa meal amendments followed that observed by Zakaria et al. (1980) in that the greatest amount of ammonia was generated by soybean meal amendment followed by linseed and cottonseed, respectively, and that ammonia was not detected in the control or alfalfa meal amended soils. Germinability of sporangia of *P. ultimum* on the surface of amended soil was inversely correlated with the concentration of ammonia generated. One percent (w/w) soybean meal amendment resulted in substantial reductions of *P. ultimum* and *T. basicola* populations in infested soil in both closed and open containers. With the reduced population density of *P. ultimum* in closed containers, a corresponding reduction of disease severity was observed. *Pythium ultimum*-infested soils showed a rapid population increase two days after soybean meal was added under the more aerobic conditions of the open containers; *P. ultimum* appears able to rapidly colonize the soybean meal as an energy source. Considering the aggressive colonizing behavior of *P. ultimum* in soil and its ability to rapidly form new sporangia and oospores in culture, the subsequent decrease in population density was probably not a result of hyphal autolysis due to exhaustion of substrate before formation of new sporangia or oospores.

More likely, with the passage of time, ammonia generated from the microbial decomposition of soybean meal, had accumulated to concentrations toxic to P. ultimum. This may explain why population levels were higher in open containers and why population reductions occurred more slowly with lower concentrations of soybean meal.

Thielaviopsis basicola did not appear to colonize the soybean meal amendment in either open or closed containers since the populations remained stable in the controls throughout most of the experiments. In the amended soils, the rate of population reduction was faster in the closed containers probably reflecting the reduced loss of volatile ammonia.

Chapter 3

EFFECT OF UREA, NITRATE, AND AMMONIA ON FUNGAL VIABILITY IN THE LABORATORY

3.1 INTRODUCTION

Soybean meal reduced populations of soil-borne pathogens most rapidly and to the greatest extent at high concentrations. Pythium ultimum was able to grow on the amendment and increase its population in open containers. While soybean meal eventually reduced the P. ultimum populations below the controls in open containers, in field conditions the population density may remain elevated long enough to present a serious potential for increased disease incidence. Therefore, urea was examined as an alternate ammonia-yielding amendment because it is easier to handle, readily available, relatively inexpensive, and is an improbable energy substrate for fungi.

The effect of urea in soil on the sclerotia of Macrophomina phaseolina, chlamydospores of Thielaviopsis basicola, and the sporangia of P. ultimum was examined. The effect of urea itself on these propagules in vitro also was studied. Ammonia and carbon dioxide are the principle hydrolysis products from urea. In soil ammonia can be further converted to nitrite and nitrate. For this reason, the effect of

nitrate on these propagules was also examined in vitro.

3.2 MATERIALS AND METHODS

3.2.1 Fungi and preparation of inocula.

Pythium ultimum was grown on hemp seed broth (Schlub and Schmitthenner, 1978) or on 0.25 or 0.1 strength carrot broth for 10 or more days at 24-26°C. Sporangia were obtained as described previously. T. basicola was cultured on PDY broth at 24-26°C for 1 1/2 months and inocula for soil was prepared as described previously. Macrophomina phaseolina was cultured in 80 ml of PD-broth in Roux bottles at 24-27°C for 2-4 weeks. Mycelial mats of M. phaseolina were collected, rinsed with distilled H_2O, and dried with forced air. The dried mats were then crushed manually and passed through a 177 µm screen to partially separate the sclerotia. The sclerotia were stored at 24-27°C and added to soil as needed.

3.2.2 Assays for fungal populations.

Populations of P. ultimum were determined either by a modification of the method of Stanghellini and Hancock (1970) or by the 'well method' (Chun and Lockwood, 1982). These methods are described in detail in chapters 4 and 5. To assay for T. basicola, the soil was suspended in 0.2% water agar and shaken for 1 h before dilutions were made in 0.2% water agar. TBM-C medium was poured into plates containing soil dilutions to estimate populations of T. basicola

(Maduewesi et al., 1976). Macrophomina phaseolina populations were based on numbers of viable sclerotia recovered from soil using sucrose flotation (Chun and Lockwood, 1982). The method is described in greater detail in chapters 4 and 5.

3.2.3 Test of other high nitrogen containing compounds.

Soil was infested with P. ultimum, then amended with 1% (w/w) soybean meal, Difco Bacto-Peptone, Difco Bacto-Egg Albumen (soluble), and urea. The soil moisture was made up to -0.1 bars matric potential and unamended soil was used as the control. The soils were kept in sealed 11 x 18 cm pint size plastic bags in styrofoam cups. After 6 days the soils were assayed for Pythium.

3.2.4 Effect of urea amendment on the soil populations of Pythium ultimum and Macrophomina phaseolina.

Artificially infested sandy loam soil was brought up to -0.1 bars matric potential and amended with 1.0% urea (w/w) which is equivalent to about 10 tons per acre. After 9 days, the soil was assayed for P. ultimum (Stanghellini and Hancock, 1970). In other experiments, powdered urea was added to the soil at 0.05, 0.1, and 1.0% (w/w). The Pythium population was assayed using the 'well method'.

The effect of urea amendment was tested in soil infested with sclerotia of M. phaseolina. Infested sandy loam soil was brought up to -0.1 bars matric potential and amended with 0.1, 0.5, and 1.0% (w/w) powdered urea. Four replicates were used per treatment and the soil

was incubated in sealed 11 x 18 cm plastic pint size bags in styrofoam cups. The bags and cups were enclosed in a second plastic bag to retard evaporation and gas exchange.

3.2.5 Urea as a toxic agent to sporangia of Pythium ultimum, sclerotia of Macrophomina phaseolina, and endoconidia and chlamydospore of Thielaviopsis basicola.

To determine whether urea itself was toxic to the fungal propagules used in laboratory or field studies, several assays were performed wherein the fungal propagules were exposed to urea and tested for viability.

Sporangia of P. ultimum were collected on 15 or 20 um filters, rinsed, and resuspended in 100 ml 0.2% water agar. This stock suspension was adjusted to give approximately 100 sporangia/ml. Water agar plates containing 0, 0.12, 0.25, 0.5, and 1.0% urea were freshly prepared. Wells were made in each plate as described in chapters 4 and 5 for the 'well method' for assaying P. ultimum. One ml of the sporangial suspension was deposited in the wells of 3 to 4 plates (more plates were used than in the soil assay procedure to reduce the diluting effect of the suspension on the urea in the agar) and the plates were incubated for 18 h before the hyphae emerging from the cylindrical walls were counted. Water agar plates containing urea also were inoculated with mycelium of P. ultimum to examine the effect of urea on growth.

Sclerotia of M. phaseolina were suspended in 0.2% water agar. One

ml of the suspension was dispensed into plastic petri dishes. Freshly prepared selective medium (Papavizas and Klag, 1975), augmented with 0, 0.12, 0.25, 0.5, or 1.0% urea, was poured into each plate. The plates were incubated for 7-10 days at 30° C before colonies were counted.

Conidia and chlamydospores of T. basicola were collected and cultured on medium augmented with 0, 0.12, 0.25, 0.5, and 1.0% urea. T. basicola was cultured on TBM-C medium without antibiotics or oxgall (Maduewesi et al., 1976), at 24-26°C for two months. Endoconidia were collected by flooding the plates with distilled water, passing the suspension through a 35 µm nylon filter, and pelleting the endoconidia by centrifugation for 10 min at 3100 X g. The endoconidia were resuspended and pelleted several times before a stock suspension was made up in 0.2% water agar (100-200 CFU/ml).

To collect the chlamydospores, culture plates were first flooded with distilled water which was decanted several times to remove endoconidia. The plates were then flooded and scraped with a glass rod to dislodge the chlamydospores. Released fungal material was then filtered through a 35 µm nylon filter. The retained material was rinsed with distilled water and permitted to dry in order to destroy remaining endoconidia (an earlier test showed that only 0.4% of endoconidia survived mild desiccation). The dried chlamydospore mass was then homogenized in an Omni-mixer for five minutes at 11,200 rpm. The chlamydospores were collected by centrifugation and resuspended to make a stock suspension in 0.2% water agar (100-200 CFU/ml).

One ml of either the chlamydospore or endoconidial stock suspension

was dispensed into petri dishes. Twenty ml of TBM-C medium supplemented with urea was poured into each plate. The plates were incubated at 24-26°C for 11 days before the numbers of colonies were counted.

3.2.6 Test for urease production.

Since ammonia is derived from the degradation of urea by urease in the soil, the ability of the fungi used in the study to produce urease was determined. Christensen urea agar plates (Smibert and Krieg, 1981), with 2% urea and without urea, were inoculated with mycelia of T. basicola, P. ultimum, C. victoriae, and M. phaseolina. The urease reactions were read after 4 days. The colony sizes of the fungi on plates with and without urea also were measured.

3.2.7 Nitrate as a toxic agent to sporangia of Pythium ultimum, sclerotia of Macrophomina phaseolina, chlamydospores of Thielaviopsis basicola in vitro.

The hydrolysis of urea to ammonia in soil leaves open the potential for further conversion to nitrite and nitrate. The effect of nitrate on the fungi used in the study was determined. Nitrite was not studied since it is short-lived in soil and is converted to nitrate. The concentrations used were 0, 0.18, 0.35, 0.7, 1.4, and 2.8% sodium nitrate which contained approximately the same amount of nitrogen as the urea concentrations tested. The procedures used were similar to those for urea. The water agar plates supplemented with 2.8% sodium nitrate and inoculated with P. ultimum were incubated an additional two days and

examined for hyphal growth.

3.2.8 Nitrate as a toxic agent to conidia of Cochliobolus victoriae in soil.

An experiment was done to determine if nitrate was as toxic to conidia of C. victoriae as ammonia generated from urea. Sandy loam soil which had been held at -0.1 bars matric potential was amended with 0, 1, or 5% urea (w/w) or with 2.8 or 14% sodium nitrate (w/w) and then wetted to create a thin surface film. Membrane filters containing conidia of C. victoriae were placed on the surface of the soils. Twenty-four hours later the membranes were removed and placed on sterile distilled water for germination. After incubation on sterile water for 24 h, the membranes were stained with a phenolic rose bengal solution and percentage germination was determined by counting 100 spores per membrane.

3.2.9 Ammonia as the toxic agent generated from urea.

A preliminary experiment was conducted to determine whether ammonia was the agent responsible for the mycotoxic response observed in the field and in the laboratory when urea was applied to soil. Sclerotia of M. phaseolina were suspended in solutions containing urea and urease. Over time, the ammonia concentration was monitored, pH was determined, and aliquots of the suspension were removed and sclerotia tested for viability.

M. phaseolina isolate no. 4 was cultured on PDA for one month at

25-27°C. The fungal culture was homogenized in 250 ml cold sterile distilled water for 10 min at 8,000 rpm in an Omni-mixer. The homogenate was transferred to a large volume of cold sterile distilled water and left to settle for 10 min after which the liquid portion was aspirated off leaving only the sclerotia. This was repeated five more times with a 5 min sedimentation time, then once more with a one min sedimentation time. The sclerotia were suspended in 0.1% water agar and kept at 4°C until use.

At the start of the experiment, two ml of the sclerotial suspension and 1 ml of either the active or inactive enzyme solution were added to the reaction chambers, which contained 200 ml of a solution of 5% urea and 0.2% water agar in 0.1 M potassium phosphate buffer (pH 6.5). The active enzyme solution consisted of 2 units of urease per ml (Sigma Chemical Co., St. Louis, Missouri) in 1% EDTA buffered in 0.1 M potassium phosphate (pH 6.5). The inactive enzyme consisted of enzyme solution which had been placed in a steamer at 100°C for 10 minutes.

The reaction chambers were sampled immediately after all components were combined and thereafter 1, 2, 3, 5, 7, 19, 24, and 50 h later. Viability was determined by mixing 0.5 ml from each chamber with PDA at 42°C and observing the plates after 2-3 days. Ammonia concentration was determined by using the Berthelot reaction (phenate-hypochlorite method) (Kaplan, 1969) and pH's were determined with a glass pH electrode (Corning, 476223).

3.2.10 Effects of urea in soil on pH, ammonia concentration, microbial populations, and seed germination.

Large clay pots, 27 x 25 cm diam., set on clay saucers, were filled with 4.6 kg of either Boyer sandy loam soil or Marlette loamy sand. The pots were left to air-dry in the greenhouse for 1 week before solutions of urea or water alone was applied as a drench. The amount of urea added brought the soil urea to 0, 0.1, or 1.0% (w/w). The pots were watered daily with about 400 ml of water. The odor of ammonia was detectable as early as 3 days after the urea drench.

After a month, soil was removed from each pot for determinations of pH, microbial populations, and seed germination. For pH determination, 10.0 g of soil was mixed [1:2 (w/w)] with 0.0.1 M $CaCl_2$ for 30 min. then left undisturbed for 30 min., then read with a pH meter. Soil actinomycete counts were made using chitin agar (Hsu and Lockwood, 1975). The aerobic bacterial population was determined in trypticase soy broth agar with 50 ppm PCNB (Farley and Lockwood, 1965). Fungal populations were determined in PDA supplemented with 250 mg of chloramphenicol and 0.5 ml of TMN detergent (Union Carbide, New York, N.Y.). Seed germination was determined by placing 50 g of soil into large plastic petri dishes, 147 x 15 mm, along with seeds which had been previously soaked in water for an hour. The plates were incubated for 4-5 days at 25°C. The soil was moistened as needed. Forty soybean seeds (var. 'Hark'), 25 corn seeds (W64A from J. Clayton, Michigan State University), 50 wheat seeds (var. 'Tecumseh' from A. Ravenscroft, Michigan State University), and 20 kidney bean seeds (from F. Saettler,

Michigan State University) were used in the soil from each pot.

3.3 RESULTS

3.3.1 Effect of compounds with high nitrogen content in soil on *Pythium ultimum*.

After 6 days incubation with 1% peptone, egg albumin, urea or soybean meal, the soils were assayed for *P. ultimum*. When the bags were opened, the odor of ammonia, as well as other volatile products was apparent. In the soils amended with peptone, egg albumin or urea, *Pythium* was not detected. In the control soil, the *P. ultimum* population was 23 ± 32 (s.e.) CFU/g. In soil amended with soybean meal, the population was 2650 ± 4547 (s.e.) CFU/g.

3.3.2 Effect of urea amendment on the soil populations of *Pythium ultimum* and *Macrophomina phaseolina*.

A preliminary experiment with soil amended with 1% urea was assayed for *P. ultimum* after 9 days. The population of *P. ultimum* was 238 ± 40.8 CFU/g in the unamended controls and could not be detected in the urea-amended soil. In another experiment, when the soil was assayed for shorter time periods using different urea concentrations (Table 7), *P. ultimum* could not be detected 2 days after 1% urea was applied. With 0.1% urea, the population dropped substantially after 2 and 3 days. The 0.05% urea amendment was not effective in reducing the population of *P. ultimum*.

TABLE 7. *PYTHIUM ULTIMUM* POPULATIONS IN SOIL AT DIFFERENT TIME PERIODS AFTER AMENDMENT WITH UREA IN CLOSED CONTAINERS

	CFU/g ± s.e.			
	Percent urea (w/w)			
Days	0	0.05	0.1	1.0
0	2,280 ± 218	2,280 ± 218	2,280 ± 218	2,280 ± 218
2	2,908 ± 586	2,460 ± 242	1,660 ± 269	0
4	2,720 ± 412	2,220 ± 128	292 ± 109	0
6	2,360 ± 441	1,860 ± 218	80 ± 56	---
8	2,200 ± 141	2,440 ± 194	122 ± 79	---
10	2,360 ± 336	2,840 ± 540	62 ± 62	---

Substantial reductions in the population of M. phaseolina were observed 2-3 days after amending soil with 0.5 and 1% concentrations of urea (Table 8.). The population in soil amended with 0.1% urea differed significantly from that in untreated soil only after 8 days.

TABLE 8. MACROPHOMINA PHASEOLINA POPULATIONS IN UREA-AMENDED SOILS IN CLOSED CONTAINERS

	CFU/g ± s.e.			
	Percent urea (w/w)			
Days	0	0.1	0.5	1.0
0	220 ± 42	220 ± 42	220 ± 42	220 ± 42
1	375 ± 41	235 ± 64	260 ± 22	115 ± 33
2	280 ± 71	430 ± 13	128 ± 31	0
3	221 ± 79	252 ± 54	0	0
4	470 ± 78	335 ± 108	25 ± 25	0
5	460 ± 60	270 ± 13	7.5 ± 2.5	1 ± 2
6	345 ± 42	335 ± 42	0.5 ± 0.5	0
8	447 ± 53	205 ± 28	0.5 ± 0.5	0

3.3.3 Toxicity of urea to sporangia of Pythium ultimum, sclerotia of Macrophomina phaseolina, and endoconidia and chlamydospore of Thielaviopsis basicola.

No visual differences were observed in mycelial growth of P. ultimum on plates supplemented with urea from 0 to 1.0%. Where sporangia were applied to urea-supplemented plates, the number of viable propagules was reduced slightly with 1.0% urea, but not at lower concentrations (Table 9). This small reduction in numbers probably represented an osmotic inhibition or a salt ion toxicity on the fungus.

No effect on viability of M. phaseolina sclerotia was observed in the urea-supplemented plates; on the contrary, the number of colonies actually increased as urea concentration increased (Table 9). However, at the higher concentrations of urea, colonies were smaller and not as darkened as those from sclerotia exposed to the lower concentrations of urea. This inhibitory effect also may have been the result of an osmotic or salt effect. The smaller sizes of the colonies could also account for the higher counts observed with the higher concentrations of urea since this permitted colonies to express themselves which would otherwise be obscured by larger, faster growing neighboring colonies.

No substantial differences were observed between the urea supplemented plates and the controls with regard to colony counts of T. basicola, arising from either endoconidia or chlamydospores (Table 9).

TABLE 9. EFFECT OF UREA-SUPPLEMENTED MEDIA ON FUNGAL PROPAGULES

	CFU/ml ± s.e.			
	P. ultimum	*M. phaseolina*	*T. basicola*	*T. basicola*
% Urea	sporangia	sclerotia	endoconidia	chlamydospores
0	200 ± 25	132 ± 4	38 ± 0.6	55 ± 5.5
0.12	193 ± 14	129 ± 7	42 ± 4.3	52 ± 3.6
0.25	195 ± 5	140 ± 4	37 ± 3.1	48 ± 3.1
0.5	190 ± 14	153 ± 7	40 ± 4.2	51 ± 3.0
1.0	163 ± 5	170 ± 5	33 ± 3.4	44 ± 3.9

3.3.4 Urease production by *P. ultimum*, *T. basicola*, *M. phaseolina*, and *C. victoriae*.

All four fungi showed reduced colony size when grown on the urea-augmented plates as compared to controls. *Cochliobolus victoriae* and

T. basicola were urease-positive while P. ultimum and M. phaseolina were urease-negative. The ability to produce urease did not appear to differentially decrease growth by C. victoriae as compared with M. phaseolina and P. ultimum. Colony growth of T. basicola was very slow on Christensen agar without urea and its much slower growth in the presence of urea may have been partially due to the ammonia produced. The slower growth of the other three fungi in the presence of urea was probably due to osmotic or salt inhibition (Table 10).

TABLE 10. UREASE PRODUCTION AND AVERAGE COLONY DIAMETER OF FOUR FUNGI ON CHRISTENSEN UREA AGAR WITH 2% UREA OR WITHOUT UREA AFTER 4 DAYS

		Colony diameter	
Fungus	Urease	Without urea (cm ± s.e.)	With urea (cm ± s.e.)
C. victoriae	+	6.3 ± 0.2	2.3 ± 0.1
T. basicola	+	1.5 ± 0.03	< 0.1[x]
M. phaseolina	-	4.7 ± 0.3	1.1 ± 0.2
P. ultimum	-	8.0 ± 0	2.7 ± 0.3

[x]Growth appearing only as fringe around inoculum piece

3.3.5 Toxicity of nitrate to sporangia of Pythium ultimum, sclerotia of Macrophomina phaseolina, and chlamydospores of Thielaviopsis basicola.

The number of colonies formed by P. ultimum decreased with 1.4 and 2.8% sodium nitrate, the highest concentrations used (Table 11). As a test for inhibitory vs. lethal effects, the water agar plates supplemented with 2.8% urea were incubated for an additional two days. The mean percentage of wells/plate showing growth was 49.2 ± 3.2% as compared to 0 initially, suggesting that sodium nitrate may be inhibitory rather than toxic, in contrast to the lethal effect of ammonia at the same concentration of nitrogen.

No toxic effect of nitrate on the sclerotia of M. phaseolina was apparent; instead, the number of colonies tended to increase slightly and the colony size to decrease as the concentration of sodium nitrate increased (Table 11). The smaller colony size probably accounted for the higher counts, at high sodium nitrate concentrations through decreased interference.

Chlamydospores of T. basicola failed to germinate and form colonies at 1.4 and 2.8% sodium nitrate (Table 11). At the lower concentrations, no differences between the sodium nitrate-supplemented plates and the controls were observed. The colony size of T. basicola decreased with increasing concentration of sodium nitrate. Average colony sizes as determined in one representative trial were 6.0 ± 0.3,

2.8 ± 0.1, 1.9 ± 0.1, and <1 mm, respectively for 0, 0.18, 0.35, and 0.7% sodium nitrate.

TABLE 11. EFFECT OF DIFFERENT CONCENTRATIONS OF SODIUM NITRATE IN AGAR MEDIA ON GROWTH OF FUNGAL PROPAGULES

	CFU/ml ± s.e.		
Sodium nitrate, %	*P. ultimum* sporangia	*M. phaseolina* sclerotia	*T. basicola* chlamydospores
0	88 ± 5.7	145 ± 6.9	125 ± 6.6
0.18	92 ± 6.1	170 ± 5.7	116 ± 5.6
0.35	88 ± 6.0	157 ± 8.7	124 ± 5.2
0.7	87 ± 5.0	170 ± 9.8	113 ± 6.0
1.4	27 ± 2.3	187 ± 6.6	0
2.8	0	157 ± 7.6	0

3.3.6 Toxicity of nitrate in soil to conidia of *Cochliobolus victoriae*.

No ammonia was detected in the nitrate-amended soils. The odor of ammonia was detected in the soils amended with 1% urea and was much stronger in the 5% urea-amended soils. Germination of the spores

placed on the control soils was 76.8 ± 1.4%, as compared with 67.4 ± 3.2 and 0%, respectively, for the soils amended with 1 and 5% urea, and 54.2 ± 4.6% and 37.1 ± 7.2% for the soils amended with 2.8 and 14% sodium nitrate, respectively.

3.3.7 Ammonia generated from urea as the agent toxic to sclerotia of Macrophomina phaseolina.

Very little spontaneous breakdown of urea to ammonia occurred in the reaction vessels with the inactivated urease and the pH remained stable, whereas ammonia was generated immediately after active urease was added, and increased with time (Table 12). The sclerotia of M. phaseolina in the vessels with the inactivated enzyme germinated, colonized the plates, and formed new sclerotia. Viability decreased in the reaction vessels with active urease. The samples taken at zero time and at 1 h did not appear visually different from the controls. Formation of sclerotia was delayed in the second h samples. In the third and fifth h samples no differences from the control plates were observed. In the seventh h samples, the colonies were 1/2 to 1/5 the size of those in the control plates and only 1/3 to 1/2 the plates were colonized compared with complete colonization exhibited in the controls. Only one 2-5 mm colony was observed in the 19th h plates. No colonies were observed in the 24 and 50th h samples.

TABLE 12. AMMONIA GENERATED IN REACTION VESSELS FROM UREA IN THE PRESENCE OF UREASE AT DIFFERENT TIME INTERVALS

Time, hours	Inactivated urease[x]		Active urease[y]	
	pH	NH_3(mg/L)	pH	NH_3(mg/L)
0		<1		14
1		1		125
2		<1		622
3		2		263
5	6.6	1	7.2	1521
7	6.6	2	7.4	1129
19	6.6	2	8.6	1613
24	6.6	4	8.8	1684
50	6.6	7	9.0	2653

[x]Urease inactivated by heat
[y]Urease, 2 units per ml

3.3.8 Effects of urea in soil on pH, microbial populations, and seed germination

After a month of incubation, soil pH increased and remained high with the 1.0% urea amendments but not with 0.1% urea (Table 13). The odor of ammonia was detectable three days after the urea was added and for as long as three weeks into the experiment. After a month, the

odor of ammonia was not detected in any of the pots. Germination of soybean, corn, kidney bean, and wheat seed on soil treated with 1.0% and 0.1% urea did not differ from their germination on untreated soil (Table 14). Populations of actinomycetes were lower in the urea-treated soil, the difference being greater with the higher concentration of urea (Table 15). The fungal population also decreased in urea-treated soil except that it was somewhat increased by 0.1% urea in the Marlette soil. Only the bacterial populations increased with increasing concentration of urea.

TABLE 13. SOIL pH AT INDICATED UREA CONCENTRATION (w/w) AFTER ONE MONTH INCUBATION

	pH ± s.e. (0.01 M $CaCl_2$)		
	Percent urea		
Soil	0	0.1	1.0
Boyer sandy loam	$5.4^{+0.22}_{-0.14}$	$5.2^{+0.27}_{-0.11}$	$8.0^{+0.24}_{-0.15}$
Marlette loamy sand	$4.8^{+0.04}_{-0.04}$	$4.9^{+0.21}_{-0.14}$	$8.2^{+0.09}_{-0.08}$
Mean	$5.0^{+0.16}_{-0.12}$	$5.0^{+0.16}_{-0.30}$	$8.0^{+0.13}_{-0.10}$

TABLE 14. EFFECT OF UREA-AMENDED SOIL[x] ON SEED GERMINATION

	Percent germination ± s.e.[y]		
	Percent urea (w/w)		
Seed	0	0.1	1.0
SOYBEAN	85.4 ± 3.4	87.9 ± 2.4	90.4 ± 1.2
CORN	78.0 ± 7.1	83.3 ± 3.6	84.0 ± 3.1
KIDNEY BEAN	43.3 ± 7.9	43.3 ± 5.3	39.2 ± 3.5
WHEAT	80.3 ± 2.3	63.7 ± 9.6	77.0 ± 3.6

[x]Urea-amended soil incubated one month before germination determinations were begun

[y]Percentage germination represent combined Boyer and Marlette soil results

TABLE 15. EFFECT OF UREA-AMENDED SOIL ON MICROBIAL SOIL POPULATIONS AFTER ONE MONTH INCUBATION

		CFU/g ± s.e.		
% Urea	Soil	Actinomycetes ($x10^4$)	Bacteria ($x10^6$)	Fungi ($x10^3$)
0	MARLETTE	51.6 ± 2.2	6.5 ± 0.6	26.2 ± 0.4
0.1	"	31.2 ± 3.3	10.6 ± 1.6	43.6 ± 6.3
1.0	"	4.8 ± 0.6	106.0 ± 4.0	3.2 ± 0.1
0	BOYER	69.4 ± 7.2	12.5 ± 0.7	62.6 ± 3.9
0.1	"	35.4 ± 1.8	17.9 ± 1.6	28.4 ± 2.8
1.0	"	8.4 ± 0.7	58.2 ± 4.4	19.4 ± 2.4*

*Fewer species of fungi were observed

3.4 DISCUSSION

Organic nitrogen sources other than soybean meal, such as peptone, egg albumin, and urea were capable of reducing populations of *P. ultimum* in soil. For practical considerations such as cost, ease of handling, availability, and effectiveness, urea was considered to be the most suitable alternative to soybean meal. In addition, urea did not appear to function as a food source for *P. ultimum* and so the potential danger of increased disease incidence and severity was eliminated.

The agent toxic to pathogenic fungi resulting from the addition of urea to soil was most likely ammonia. Nitrate and urea by themselves were not inhibitory or lethal to the propagules of *P. ultimum*, *T. basicola*, and *M. phaseolina* except at the highest concentrations, 1.0% urea and 1.4 and 2.8% sodium nitrate, and at these concentrations the effects were probably osmotic inhibition. The presence of urea by itself in the soil cannot account for the population reductions observed since the population assays for the fungi would remove the inhibitory effect of urea, through dilution. The argument that ammonia can account for the reduction of *P. ultimum* and *M. phaseolina* and *T. basicola* in soil was strengthened by the observation that ammonia generated from the hydrolysis of urea can account for the reduced viability of *M. phaseolina* in vitro.

The hydrolysis of urea in soil and subsequent production of ammonia

greatly upset the soil environment, as evidenced by increased pH and changes in microbial populations. Moreover, changes in soil structure and other physical characteristics of the soil such as availability of organic and inorganic nutrients, etc., may result from the increased pH and presence of ammonia. Although inhibition of germination of various seeds was not observed, plant growth may be affected by these immediate and long term changes. Further work should be directed towards the long term soil ecology, particularly in regard to microbial and soil environmental effects on growth of different plants.

Chapter 4

POPULATION REDUCTION OF *PYTHIUM ULTIMUM*, *THIELAVIOPSIS BASICOLA* AND *MACROPHOMINA PHASEOLINA* IN SOIL DUE TO AMMONIA GENERATED FROM UREA

4.1 INTRODUCTION

Nitrogen fertilizers can influence plant diseases by altering plant resistance, by directly affecting the pathogen, or by affecting the soil microbiota which may in turn influence the pathogen-host interaction (Huber and Watson, 1974). Certain nitrogenous materials showing fungicidal activity *in vitro* (Filonow and Chun, 1981; Gilpatrick, 1969a, 1969b; Schippers and Palm, 1973; Zakaria et al., 1980), have been used in attempts to control soil pathogens, with both positive (Chun and Lockwood, 1982; Tsao and Zentmeyer, 1979) and negative results (Smiley et al.,1972; Zakaria and Lockwood, 1980). Oilseed meal amendments were effective in reducing *Fusarium* chlamydospore populations in the laboratory, but not in the field (Zakaria and Lockwood, 1980). The relative effectiveness of the amendments in laboratory studies was directly correlated with the amount of ammonia produced upon decomposition of the amendment (Zakaria et al., 1980). This finding suggested the possible use of urea as an alternate source of ammonia.

The purpose of this study was to investigate the effects of soybean meal and urea on populations of Pythium ultimum Trow., Macrophomina phaseolina Goid. [=M. phaseoli (Maubl.) Ashby], and Thielaviopsis basicola (Berk. & Br.) Ferr. under field conditions, and to relate population changes to changes in soil pH, ammonia (NH_3/NH_4^+) concentration, and temperature.

4.2 MATERIALS AND METHODS

4.2.1 Fungi and preparation of inocula.

P. ultimum was isolated from soybean seedling pieces (Schlub and Lockwood, 1981). M. phaseolina isolate No. 4 was obtained from T. D. Wyllie, University of Missouri. T. basicola isolate 157 was isolated from diseased soybeans in Michigan. P. ultimum was maintained on carrot agar, M. phaseolina on potato-dextrose agar (PDA), and T. basicola on potato-dextrose-yeast agar (PDYA). Carrot agar was prepared by autoclaving 30 g of sliced carrots in 250 ml of distilled H_2O and decanting the supernatant solution into 20 g of agar in 750 ml of distilled H_2O. PDYA was made by adding 5 g of Difco yeast extract per L of PDA.

P. ultimum for infesting soil was cultured in 2 L of 0.25 or 0.1 strength carrot broth in autoclavable bags (American Scientific Products, McGaw Park, Ill. 60085). M. phaseolina and T. basicola were cultured in 500 ml of PD-broth and PDY-broth, respectively, in aluminum pans (50 x 29 x 8 cm) covered with aluminum foil, or in 80 ml of broth

in Roux bottles. All three pathogens were cultured at 24-27°C for 2-4 weeks.

Sporangia of P. ultimum, sclerotia of M. phaseolina, and chlamydospores of T. basicola were used to infest soil. Mycelial mats of P. ultimum and T. basicola were collected, rinsed with distilled H_2O, and homogenized with an Omni-mixer (Ivan Sorvall, Norwalk, CT 06856) at 6,400 rpm for 30-60 sec and 8,000 rpm for 10 min, respectively. Mycelial mats of M. phaseolina were collected, rinsed with distilled H_2O, and dried with forced air. The dried mats were then crushed manually and passed through a 177 µm screen to partially separate the sclerotia. The homogenates of P. ultimum were pooled and manually mixed directly into the Rose Lake field plots. Homogenates of all three pathogens were mixed into smaller amounts of soil for later incorporation (see below). These stock soils were kept moist at 24-27°C for at least a week to permit lysis of mycelial fragments, then were air-dried and thoroughly mixed with each other.

4.2.2 Assays for fungal populations.

Populations of P. ultimum for the Rose Lake trial were determined by the method of Stanghellini and Hancock (1970) modified as follows: soil was diluted and suspended in 0.2% water agar blanks; 1 ml of each dilution was spread over 3-10 plates by using a greater number of smaller drops per plate than were used previously (Stanghellini and Hancock, 1970), thereby improving the accuracy of the counts and accommodated a greater population range. For the microplot trials, the method was modified further to use fewer plates, to improve sensitivity

and accuracy of the assay, to afford more latitude in the incubation time, and to reduce the time required to do the assay (Chun and Lockwood, 1982). Soil dilutions were dispensed into 2.5 mm diam. wells of 2 mm depth made in 2% water agar plates (50-52 wells per plate) at approximately 0.01 ml per well. Hyphal growth from the walls of the agar wells was easily observed in the inverted plates. Hereafter, this assay will be referred to as the 'well-method'.

Populations of the three pathogens, soil pH, and ammonia concentrations were determined in the microplots. Soil samples were kept on ice until assayed, usually within 1 h after collection. Each sample was subdivided: 5 or 10 g was placed in a plastic screw-top centrifuge tube to assay for M. phaseolina, 10 g was used to assay for both P. ultimum and T. basicola, 10 g was used to determine pH, 10 g was used for ammonia concentration determinations, and 10-40 g was used to determine soil moisture content (sample dried overnight at 90°C). To assay populations of P. ultimum and T. basicola, the soil was suspended in 0.2% water agar and shaken for 1 h before dilutions were made in 0.2% water agar. TBM-C medium was poured into plates containing soil dilutions to estimate populations of T. basicola (Maduewesi et al., 1976). P. ultimum populations were estimated with the well-method.

M. phaseolina populations were based on numbers of viable sclerotia recovered from soil using sucrose flotation (Chun and Lockwood, 1982). The soil samples were dispersed in 60% (w/w) sucrose solution and centrifuged for 10 min at 3100 X g. The sclerotia were then aspirated from the top of the sucrose solution, the centrifuge cap, and the wall and lip of the tube and collected on 15 µm nylon screens. The

sclerotia were rinsed by aspiration of distilled H_2O, then were surface-sterilized in 0.5% NaOCl for 5 min, rinsed, and suspended and diluted in 0.2% water agar. Selective medium (Papavizas and Klag, 1975) was poured into plates containing the sclerotial dilutions, and the colonies counted after 7-10 days incubation at 30°C.

4.2.3 Rose Lake field trial.

Plots were located at the Rose Lake Wildlife Research Center (East Lansing, Michigan), in an Oshtemo-Boyer sandy loam soil. A total of 24 0.5 x 1.0 m plots were used, each separated by 2 m from its nearest neighbor. Three treatments were applied to the plots: (i) incorporated soybean meal (1%), (ii) incorporated soybean meal (1%) and the plot covered with a 6 mil black plastic tarpaulin, and (iii) covering with a plastic tarpaulin alone. Untreated and uncovered plots served as controls. The edges of the plastic sheets were anchored below the soil surface. Soybean meal was ground in a Wiley Mill (0.13 cm screen), and approximately 800 g was manually incorporated to a depth of 10 cm into each plot to give an approximate 1% (w/w) concentration. The treatments were randomly assigned.

Plots were infested on 14 August, 1980. On 9 September, the initial samples were collected, the soybean meal was incorporated, and the plots covered. The plots were sampled during 26 days.

4.2.4 Microplot field trials.

Three experiments were conducted at the Botany and Plant Pathology

Farm, Michigan State University, in a Marlette sandy loam soil. Two trials were done sequentially during the summer of 1981 and a third trial was done in the summer of 1982. The microplots consisted of cylindrical clay drainage tiles 20.3 cm diam. x 30.5 cm deep. Each tile was sunk about 28 cm into the field, separated by either 3.0 or 3.7 m (1981 trials) or by 2.4 m (1982 trial) from its closest neighbor. The bottom half of each tile was packed with the surrounding soil and the top half was packed with soil that had been infested with propagules of the three pathogens. The infested soil was Marlette or Boyer sandy loam (1981 trials), or Boyer sandy loam (1982 trial). The soils were left undisturbed for a week or more before urea was applied.

At the time of sampling, soil temperature was monitored, usually between 10:00 a.m. and 2:00 p.m., using 6 temperature probes buried 7-8 cm below the soil surface in selected microplots. Soil pH in trials 2 and 3 was determined in 10 g of sample mixed with 0.01 M $CaCl_2$ [1:2 (w/v)]. Soils in trials 2 and 3 were analysed for ammonia (NH_3/NH_4^+) by suspending samples in 10 ml of 0.32 M boric acid, and leaving the suspension to clear at 4 C. Microliter quantities of the boric acid solution was injected into a Varian Aerograph 1400 series gas chromatograph (Varian Instruments, Palo Alto, CA 94303) equipped with a glass column (1.83 m x 0.64 mm x 2 mm i.d.) packed with Chromosorb 103 (Johns-Manville, Denver, CO 80217) in trial 2. In trial 3, concentrations of ammonia in the boric acid solution were determined spectrophotometrically using Nessler's reaction (Fischer and Peters, 1968). Ammonia concentrations are expressed on a soil dry weight basis.

Urea granules (Mallinckrodt, Inc., Paris, KY 40361) were dissolved in water and applied as a drench to the microplots. Concentrations, based on the amount of infested soil in the microplots, were 0.1% and 1.0% (w/w) in the 1981 trials, and 0.25%, 0.5%, and 1.0% (w/w) in 1982. Water was used for controls. Treatments were randomized; 12 replicates were used for the 1981 trials and 8 for the 1982 trial. The trials began on 19 July, 24 September, and 21 July for trials 1, 2, and 3, respectively. Samples were collected after 0, 4, 12, and 31 days in the first trial; 0, 4, 9, 15, and 34 days in the second trial; and 0, 4, 9, and 40 days in the third trial.

4.3 RESULTS

4.3.1 Rose Lake trial.

Prior to application of the soybean meal populations of *P. ultimum* averaged 112 CFU/g soil. Throughout the experiment, the populations remained relatively stable in the controls (Table 16). With the addition of soybean meal, populations of *P. ultimum* increased over 10 times in covered or uncovered plots after 4 days. *P. ultimum* was also observed to actively colonize the soybean meal when samples were taken for population assay. Although the populations in soybean meal-amended soils decreased to values near those of the controls by the 26th day (uncovered plots), the failure of the amendment to markedly decrease the population led to the abandonment of soybean meal as an amendment for controlling *P. ultimum*.

TABLE 16. EFFECT OF SOYBEAN MEAL (1%, W/W) TREATMENTS ON POPULATIONS OF *PYTHIUM ULTIMUM* IN COVERED AND UNCOVERED SOILS

	P. ultimum population (CFU / g DRY WT SOIL)					
Treatment	0	4	8	13	17	26
	(days after amendment)					
Control, not covered	106a[z]	134c	77b	68b	61b	80a
Control, covered	84a	117c	62b	45b	86b	40a
Soybean meal, not covered	139a	7,191a	4,562a	3,263a	1,800a	77a
Soybean meal, covered	118a	3,364b	3,681a	1,896ab	1,774a	830a

[z]Values followed by the same letter in each vertical column do not differ significantly ($P=0.05$) according to Duncan's new multiple range test.

4.3.2 Microplot trials.

Alternate sources of ammonia were tested *in vitro* and of these, urea was chosen because of its high nitrogen content, availability, and because it did not increase populations of *P. ultimum* (Filonow and Chun, 1981). Microplots were employed in an attempt to reduce variability and to conserve inocula, which allowed inclusion of *T. basicola* and *M. phaseolina* in the experiments.

In the first trial (Fig. 1), the population of *P. ultimum* remained relatively stable in control microplots, ranging from 128-226 CFU/g throughout the 31-day period. With 0.1% urea, the population decreased to 119 and 12 CFU/g after 12 and 31 days, respectively. With 1.0% urea, the population dropped to 7 CFU/g after 4 days; by the 12th day, *P. ultimum* was not detected.

The population of *T. basicola* remained stable in the controls at about 20,000 CFU/g (Fig. 2). With 0.1% urea, the population did not decrease until the 31st day when it dropped to 932 CFU/g. The 1.0% urea treatment reduced the population to 850 CFU/g after 4 days, and to 12 CFU/g after 12 days; by 31 days, *T. basicola* was not detected.

M. phaseolina population in the controls averaged 80 CFU/g during the experiment. With 0.1% urea, the population dropped to about half that of the control (Fig. 3) by the 31st day. With 1.0% urea, the population of *M. phaseolina* dropped to about 11 CFU/g after 4 days; and after 12 and 31 days it was about 1 CFU/g.

Figure 1. Effect of 0, 0.1, and 1.0% urea amendment on soil populations of Pythium ultimum after 4, 12, and 31 days in microplots in the field. Trial 1 was conducted July 17 to August 17, 1981. Vertical bars represent one standard error.

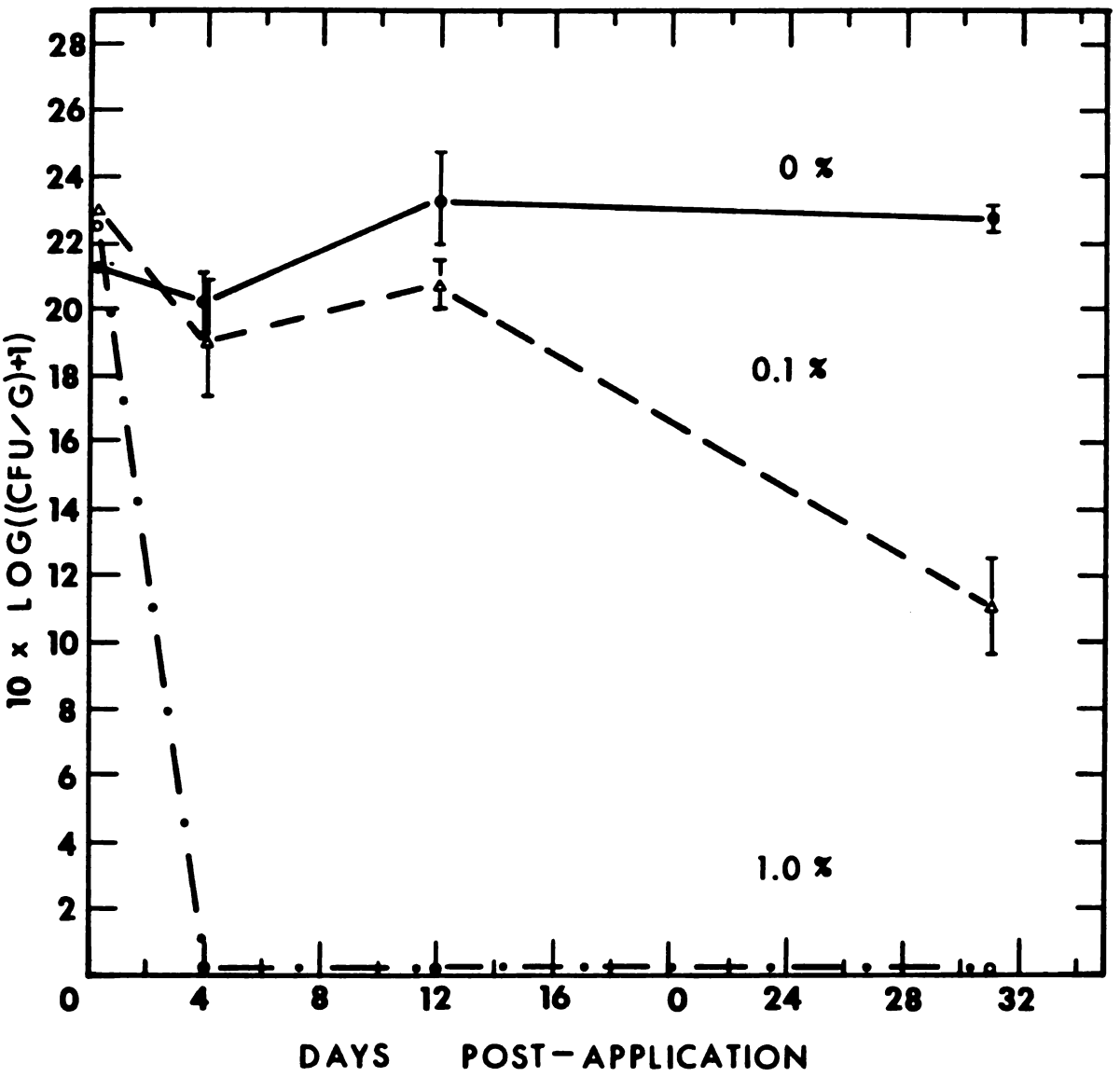

28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
10 x LOG((CFU/G)+1)
0 %
0.1 %
1.0 %
0
4
8
12
16
0
24
28
32
DAYS POST-APPLICATION

Figure 2. Effect of 0, 0.1, and 1.0% urea amendment on soil populations of Thielaviopsis basicola after 4, 12, and 31 days in microplots in the field. Trial 1 was conducted July 17 to August 17, 1981. Vertical bars represent one standard error.

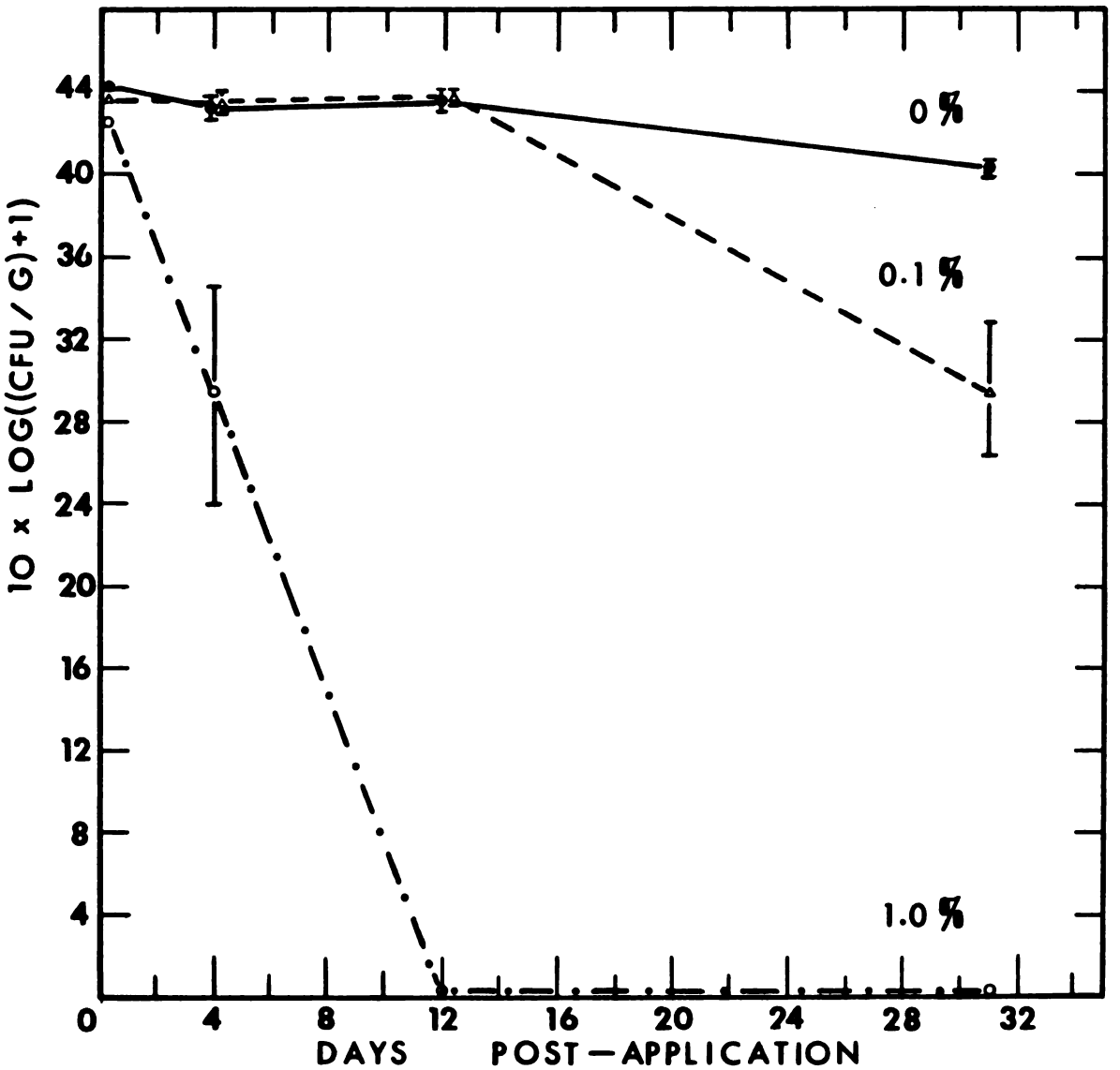
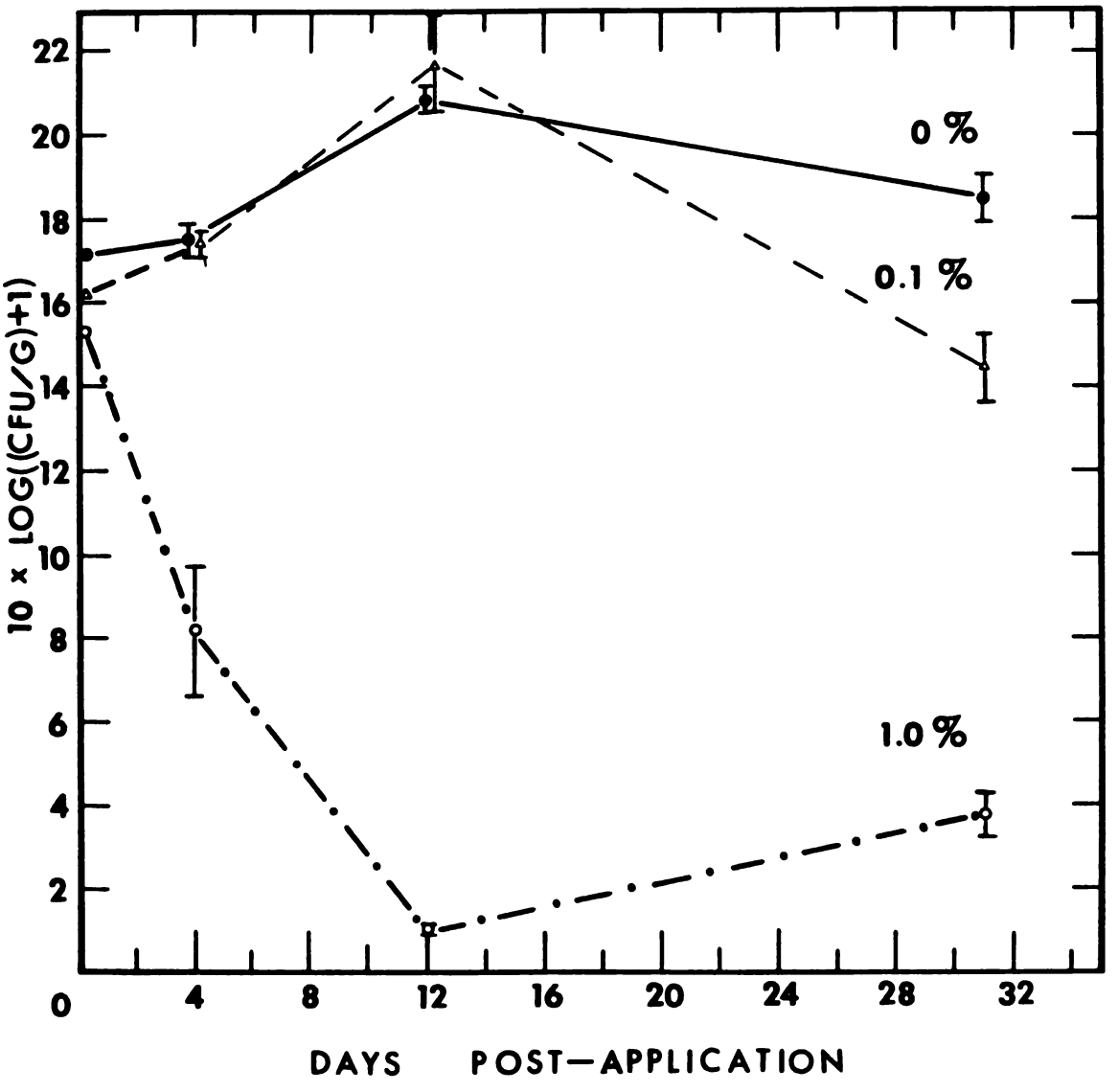

10 x LOG((CFU / G)+1)
44
40
36
32
28
24
20
16
12
8
4
0
4
8
12
16
20
24
28
32
DAYS POST—APPLICATION
0 %
0.1 %
1.0 %

Figure 3. Effect of 0, 0.1, and 1.0% urea amendment on soil populations of Macrophomina phaseolina after 4, 12, and 31 days in microplots in the field. Trial 1 was conducted July 17 to August 17, 1981. Vertical bars represent one standard error.


22
20
18
16
14
12
10
8
6
4
2
0
10 x LOG((CFU/G)+1)
0 %
0.1 %
1.0 %
0
4
8
12
16
20
24
28
32
DAYS POST—APPLICATION

During the second trial, the pathogen populations were not reduced as rapidly nor as drastically by 1.0% urea as in the first experiment; 0.1% urea did not significantly decrease the fungal populations (Fig. 4-6). Trial 2 was conducted from late September through October when soil temperatures were often 8-10 C lower than those in trial 1 (Fig. 7). Soil pH in trial 2 rose from 5.0 to a high of pH 8.7 with 1.0% urea after 4 days, then dropped to 8.0 after 34 days (Fig. 8A). With 0.1% urea the pH rose to a maximum of 6.6 after 4 days, but returned to the initial pH after 34 days. With 1.0% urea, ammonia rose to a peak of about 1900 mg/kg after 4 days, then decreased by the end of the experiment to about 500 to 600 mg/kg. Soil treated with 0.1% urea did not exceed 80 mg/kg (Fig. 8B).

Figure 4. Effect of 0, 0.1, and 1.0% urea amendment on soil populations of Pythium ultimum after 4, 9, 15, 34 days in microplots in the field. Trial 2 was conducted September 24 to October 28, 1981. Vertical bars represent one standard error.

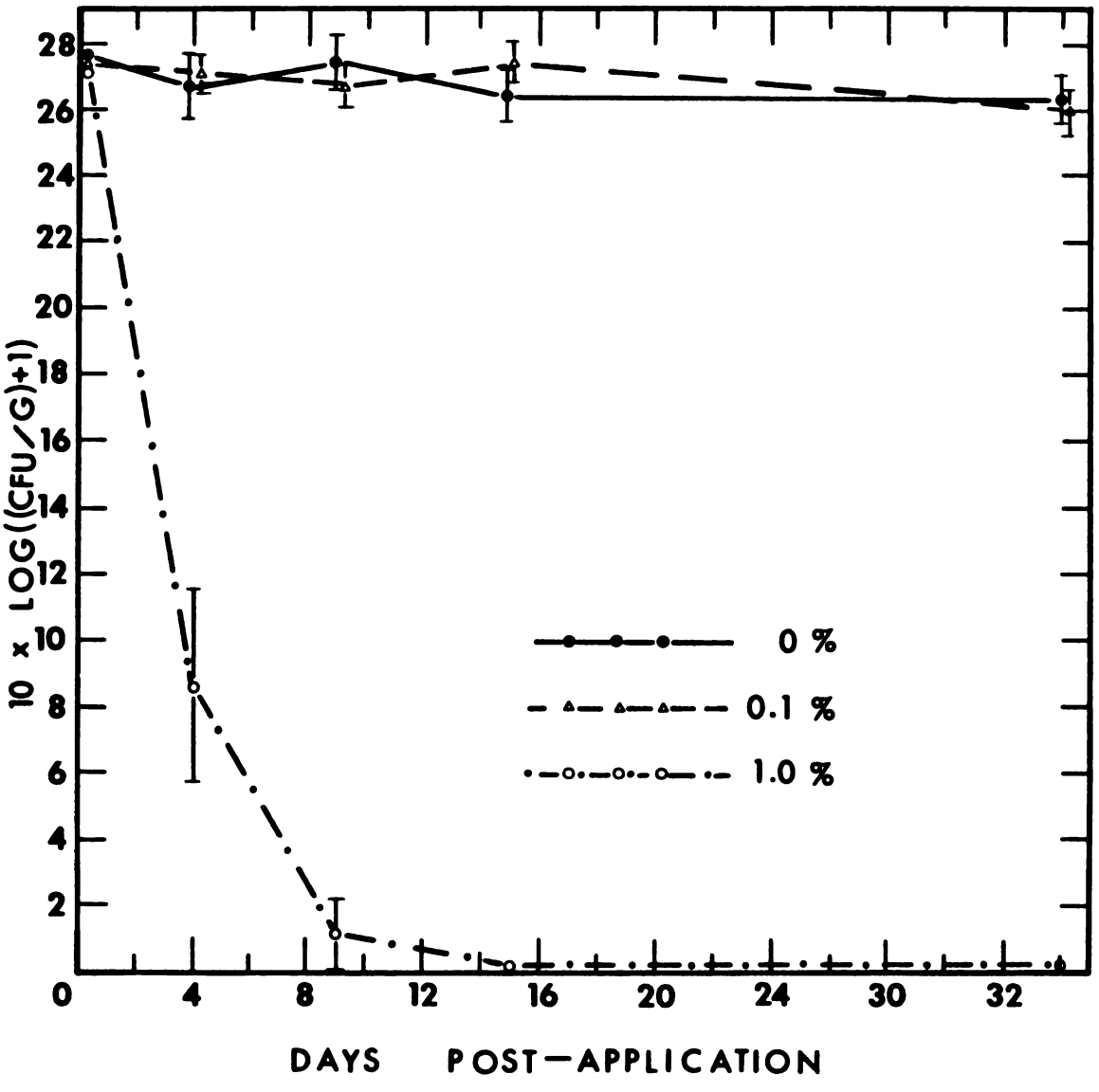

28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
10 x LOG((CFU/G)+1)
0 %
0.1 %
1.0 %
0
4
8
12
16
20
24
30
32
DAYS POST-APPLICATION

Figure 5. Effect of 0, 0.1, and 1.0% urea amendment on soil populations of Thielaviopsis basicola after 4, 9, 15, 34 days in microplots in the field. Trial 2 was conducted September 24 to October 28, 1981. Vertical bars represent one standard error.

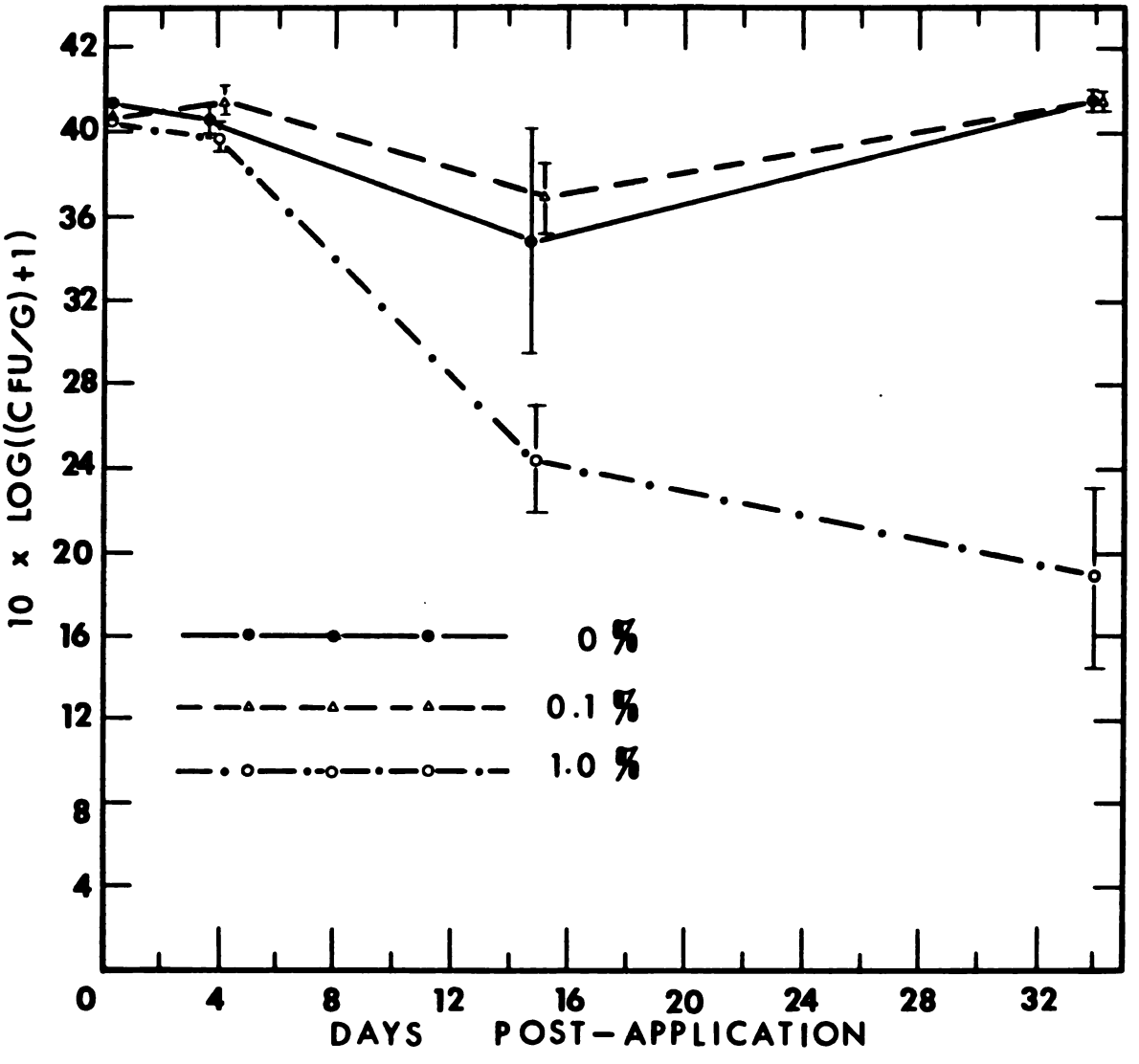

42
40
36
32
28
24
20
16
12
8
4
0
4
8
12
16
20
24
28
32
10 x LOG((CFU/G)+1)
DAYS POST-APPLICATION
0 %
0.1 %
1.0 %

Figure 6. Effect of 0, 0.1, and 1.0% urea amendment on soil populations of Macrophomina phaseolina after 4, 9, 15, 34 days in microplots in the field. Trial 2 was conducted September 24 to October 28, 1981. Vertical bars represent one standard error.

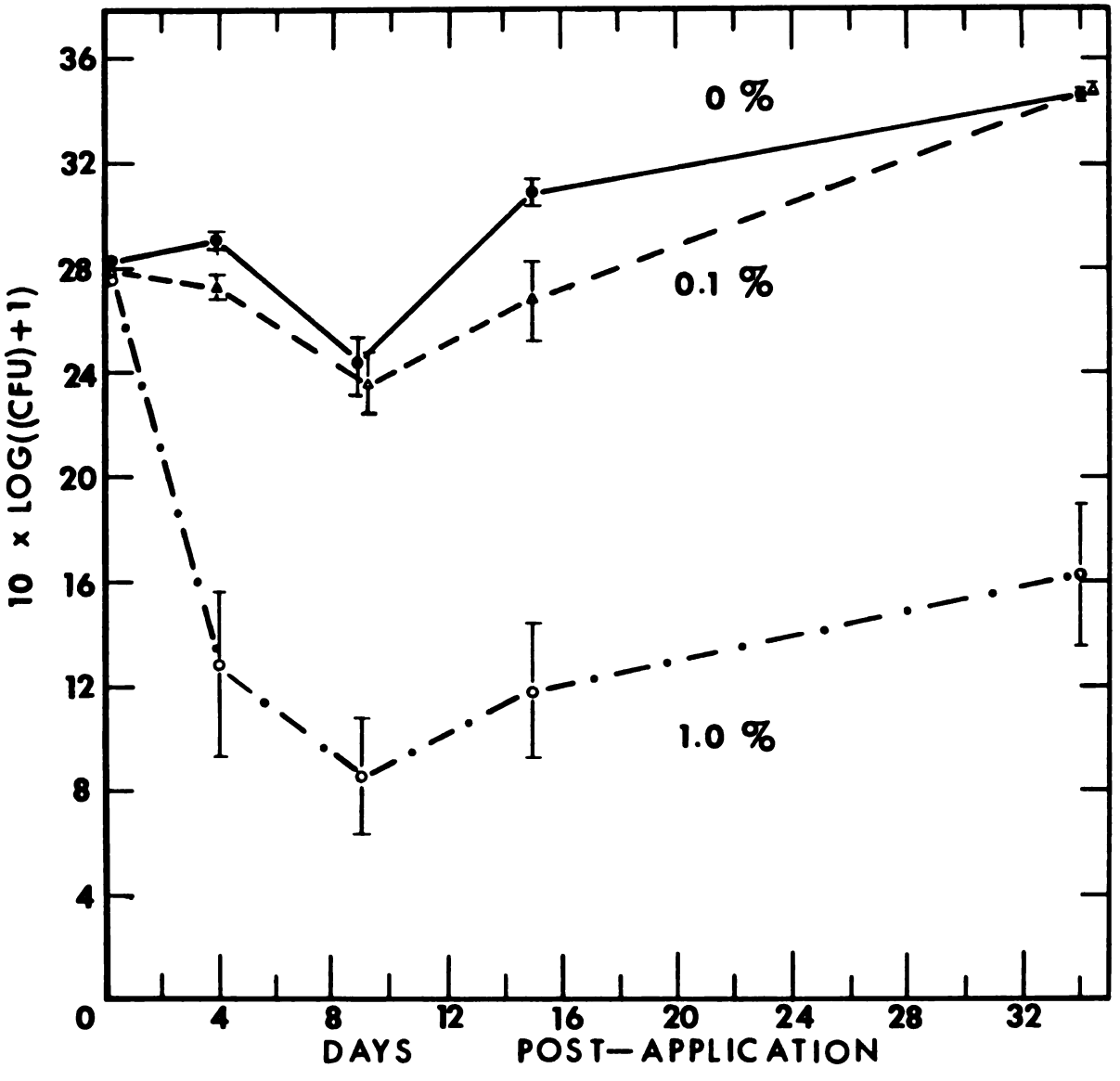

36
32
28
24
20
16
12
8
4
0
10 x LOG((CFU)+1)
0 %
0.1 %
1.0 %
4
8
12
16
20
24
28
32
DAYS POST—APPLICATION

Figure 7. Soil temperature 7-8 cm below the soil surface taken during trials 1, 2, and 3, conducted July 17 to August 17 1981; September 24 to October 28 1981; and July 21 to August 30 1982, respectively.

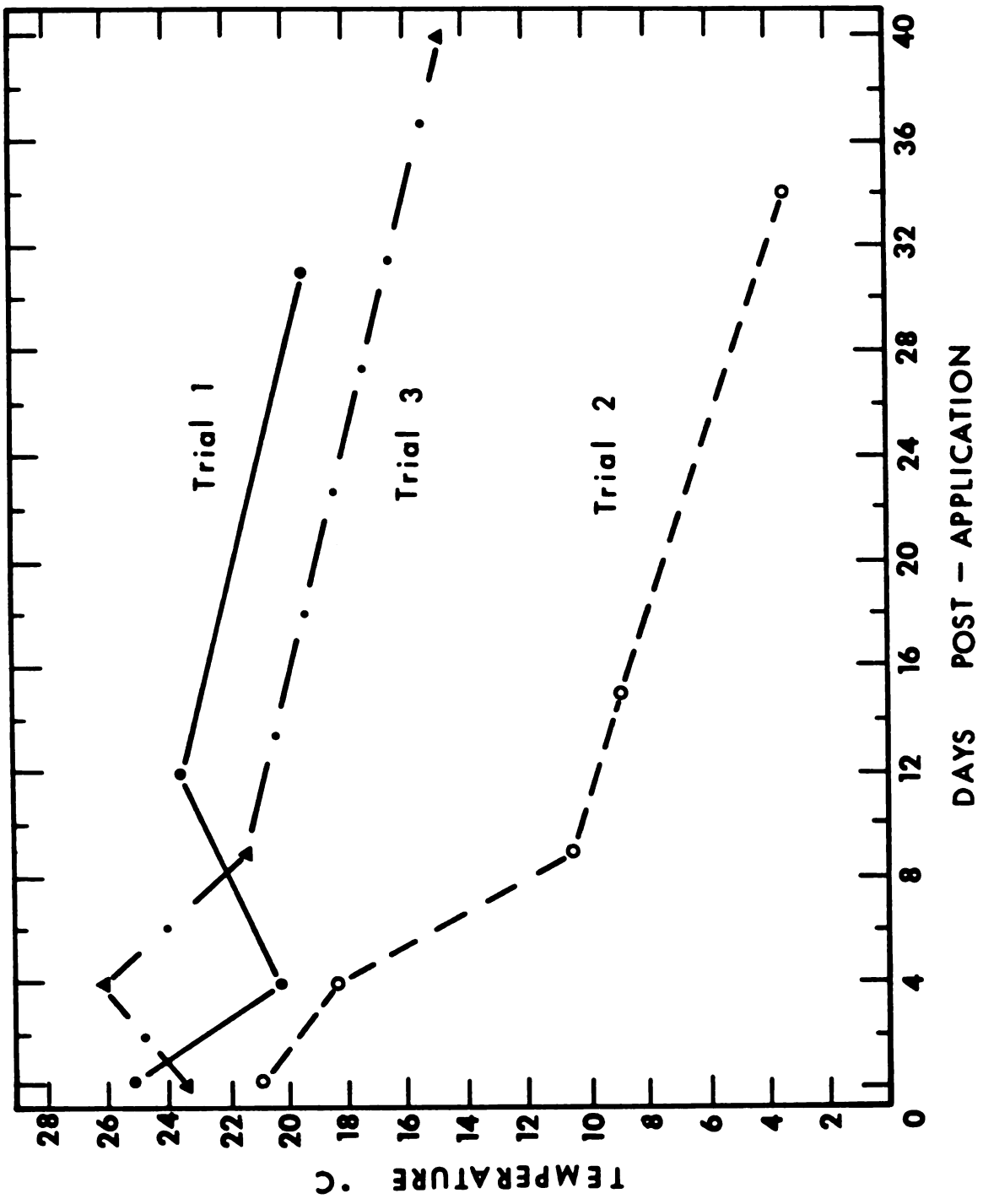
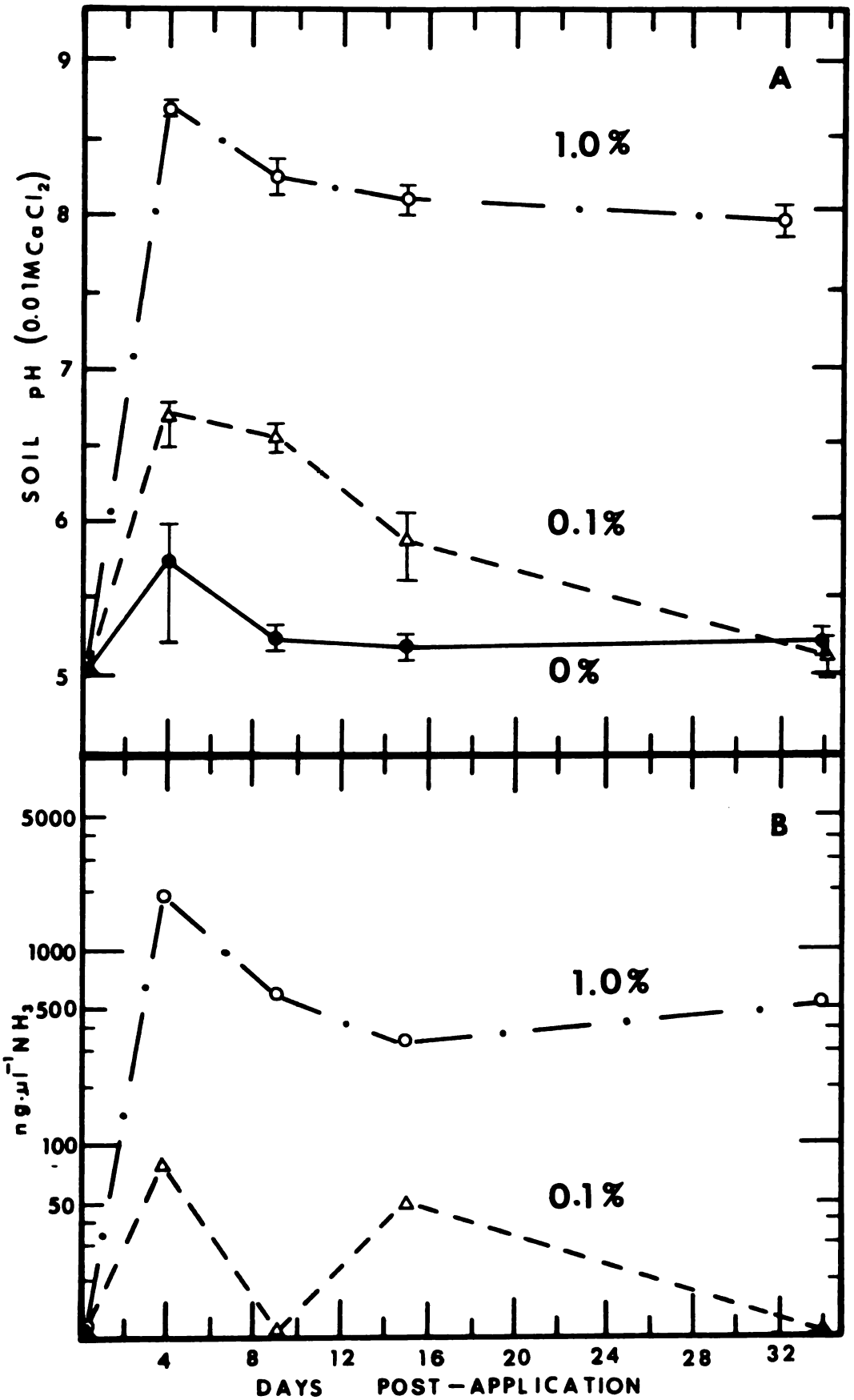
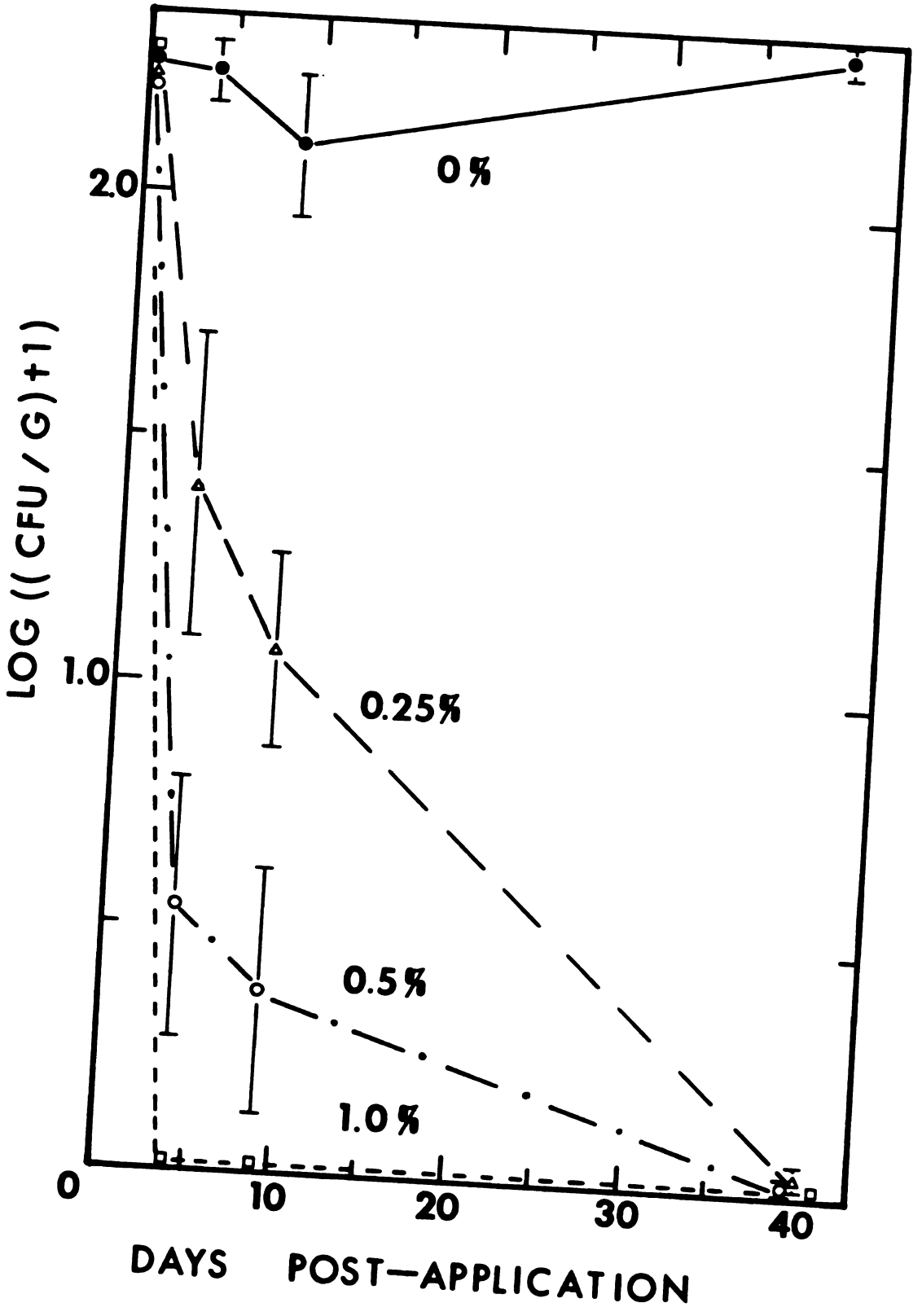

Trial 1
Trial 3
Trial 2
TEMPERATURE °C
DAYS POST – APPLICATION
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
4
8
12
16
20
24
28
32
36
40

Figure 8. Effect of 0.1 and 1.0% urea amendment of soil on (A) soil pH (vertical bars represent one standard error), and (B) soil NH_3/NH_4^+ concentration (background NH_3/NH_4^+ subtracted).


A
1.0%
0.1%
0%
SOIL pH (0.01MCaCl2)
9
8
7
6
5
B
1.0%
0.1%
5000
1000
500
100
50
ng·µl⁻¹NH3
4
8
12
16
20
24
28
32
DAYS POST-APPLICATION

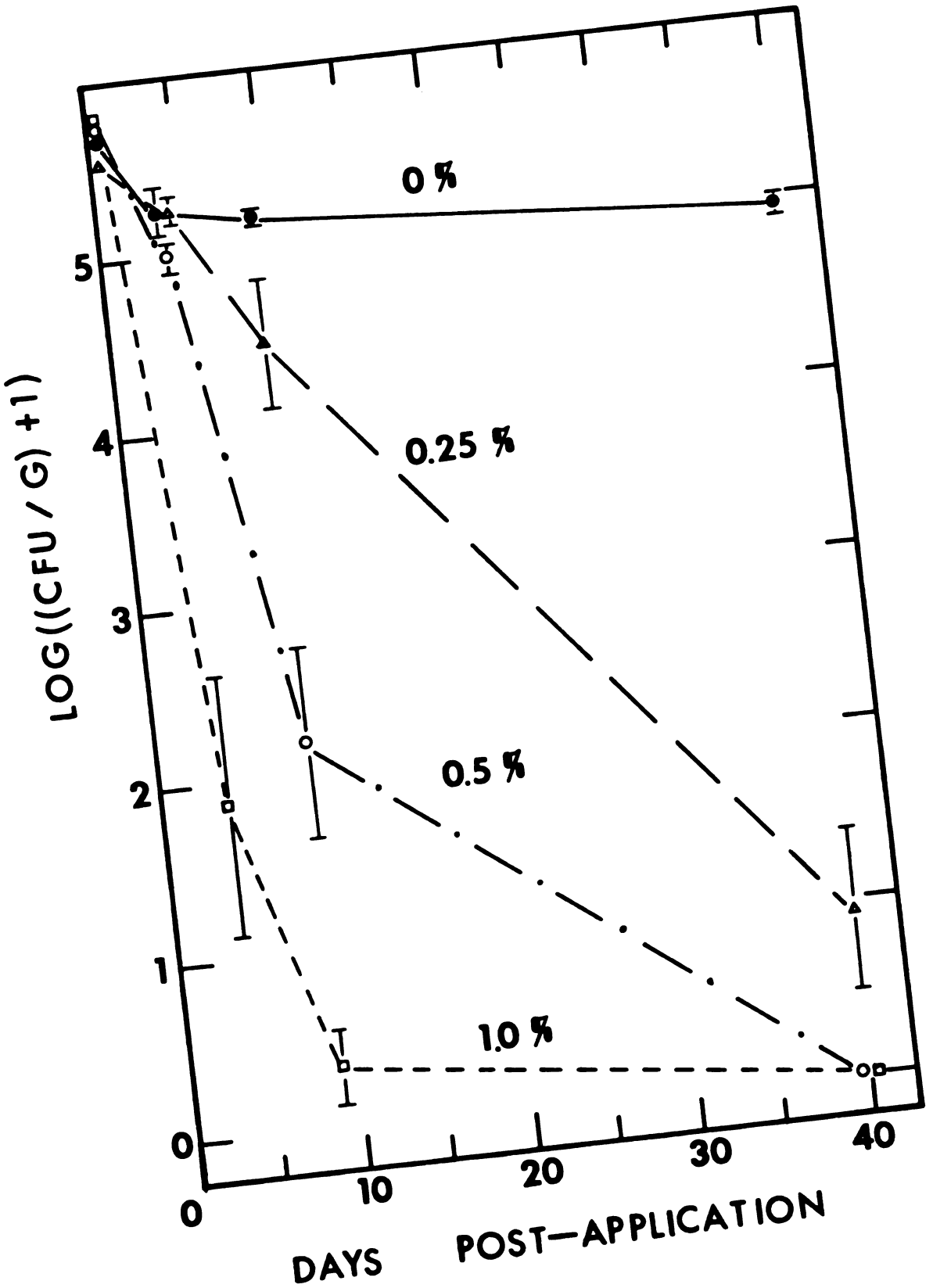
During trial 3, populations of P. ultimum in untreated soil remained stable at about 190 CFU/g during the experiment (Fig. 9). The population in soil treated with 0.25 and 0.5% urea declined sharply after 4 and 9 days and by 40 days P. ultimum could not be detected. With 1.0% urea, P. ultimum was not detected after 4 days.

Figure 9. Effect of 0, 0.25, 0.5, and 1.0% urea amendment on soil populations of Pythium ultimum after 4, 9, and 40 days in microplots in the field. Trial 3 was conducted July 21 to August 30, 1982. Vertical bars represent one standard error.


LOG ((CFU / G)+1)
2.0
1.0
0
0 %
0.25%
0.5%
1.0%
10
20
30
40
DAYS POST—APPLICATION

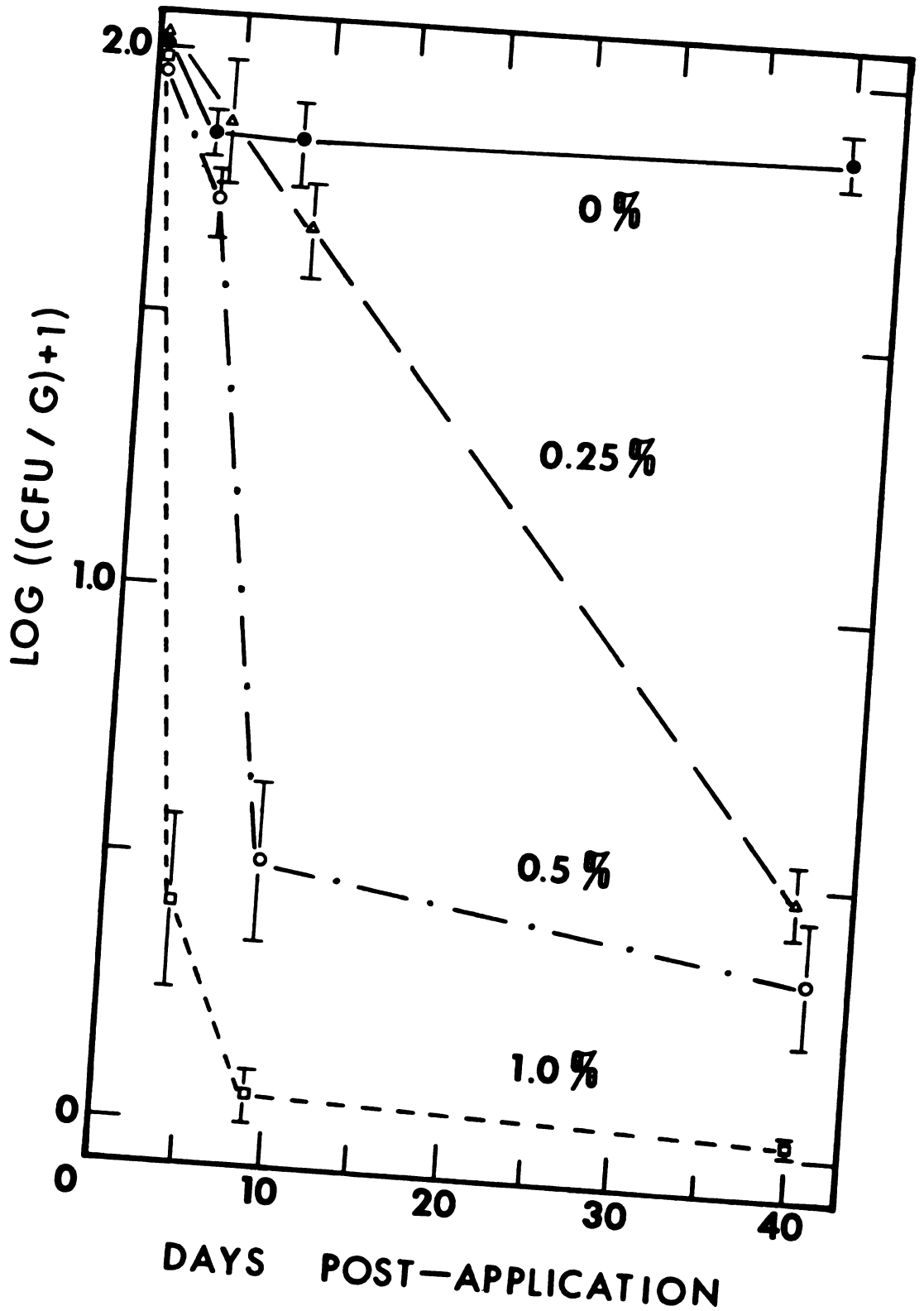
The population of T. basicola in untreated soil remained relatively constant at about 10^5 CFU/g (Fig. 10). In soil treated with 0.25% urea, the population dropped to less than 0.17 of its initial size after 9 days, and by the 40th day was barely detectable. The population in soil treated with 0.5% urea was reduced to 0.16 of its initial size after only 4 days, and to 0.01 of its initial size after 9 days; after 40 days it was not detected. When 1.0% urea was added, T. basicola could not be detected after 9 days.

Figure 10. Effect of 0, 0.25, 0.5, and 1.0% urea amendment on soil populations of Thielaviopsis basicola after 4, 9, and 40 days in microplots in the field. Trial 3 was conducted July 21 to August 30, 1982. Vertical bars represent one standard error.


0 %
0.25 %
0.5 %
1.0 %
LOG((CFU / G) +1)
0
1
2
3
4
5
0
10
20
30
40
DAYS POST—APPLICATION

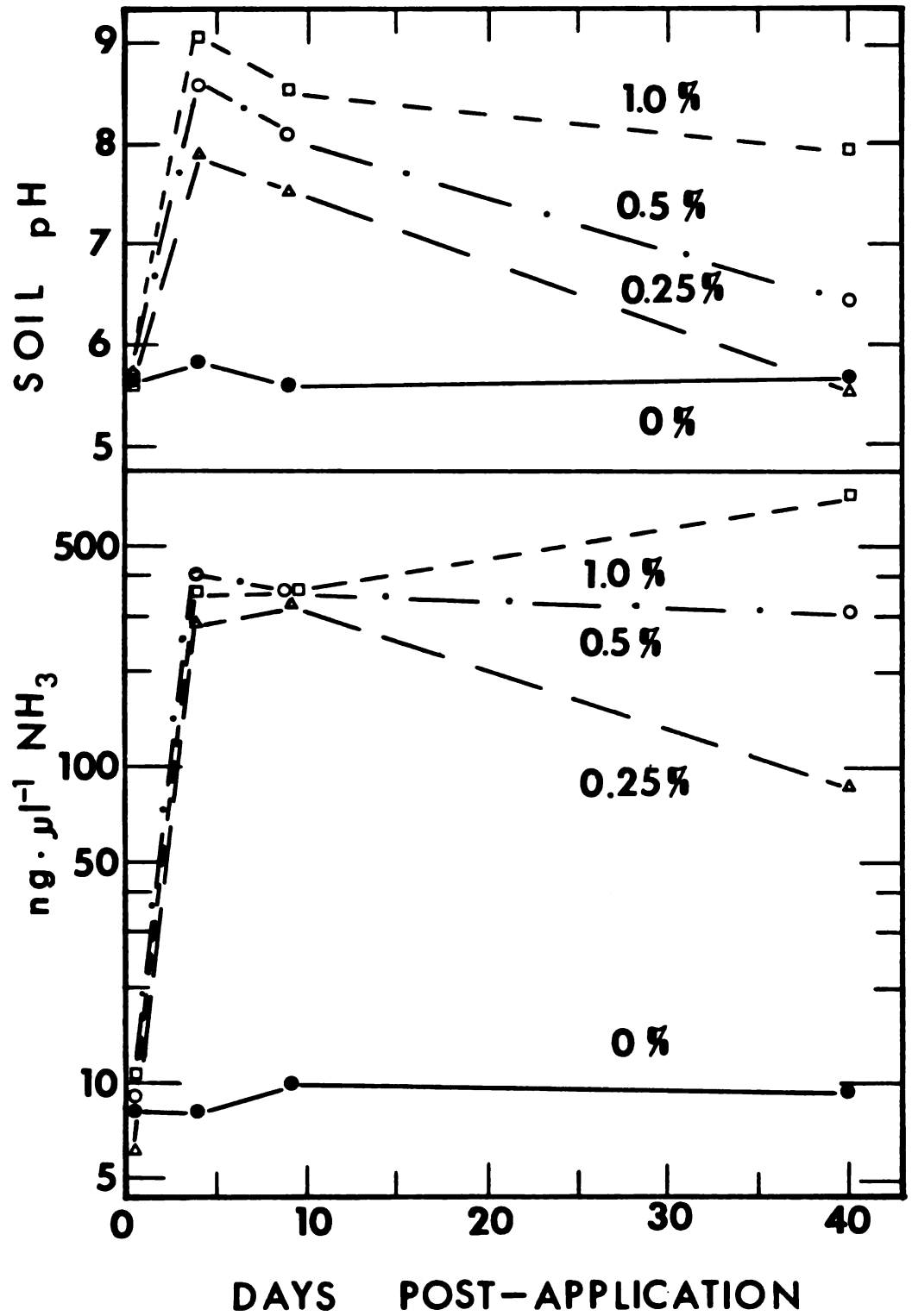
The M. phaseolina population in control soil was about 81 CFU/g throughout the experiment. The population in soil treated with 0.25% urea decreased by about half after 9 days, and to about 0.02 of the control after 40 days (Fig. 11). With 0.5% urea, the population was reduced to 0.02 of that initially by 4 days and by 40 days was barely detectable. With 1.0% urea, the population dropped to zero within 9 days.

Figure 11. Effect of 0, 0.25, 0.5, and 1.0% urea amendment on soil populations of Macrophomina phaseolina after 4, 9, and 40 days in microplots in the field. Trial 3 was conducted July 21 to August 30, 1982. Vertical bars represent one standard error.


2.0
1.0
0
0
10
20
30
40
LOG ((CFU / G)+1)
DAYS POST—APPLICATION
0 %
0.25 %
0.5 %
1.0 %

Trial 3 was run from late July until the end of August. Soil temperature ranged from a high of 26°C to a low of 15°C at the end of the experiment (Fig. 7). Ammonia levels in control soils remained constant at about 8-10 mg/kg (Fig. 12). In all treated soils, the ammonia concentration increased rapidly after only 4 days to about 300-400 mg/kg. By 40 days, it had dropped to 89 mg/kg with 0.25% urea, remained more or less constant with 0.5% urea, but had increased further with 1% urea. Soil pH rose to 8-9 by day 4 according to the urea concentration applied, then declined. Soil treated with 0.25 and 0.5% urea had returned to a pH near that of the control soils by 40 days, whereas soil treated with 1.0% urea remained at about pH 8.0.

Figure 12. Effect of 0, 0.25, 0.5, and 1.0% urea amendment of soil on (A) soil pH, and (B) soil NH_3/NH_4^+ concentration. Trial 3 was conducted July 21 to August 30, 1982.


SOIL pH
9
8
7
6
5
1.0 %
0.5 %
0.25%
0 %
ng · µl⁻¹ NH3
500
100
50
10
5
1.0 %
0.5 %
0.25%
0 %
0
10
20
30
40
DAYS POST-APPLICATION

4.4 DISCUSSION

The failure of soybean meal to reduce populations of P. ultimum in the field experiments may have been due to several factors. The pathogen was observed to colonize the soybean meal which could account for the population increases. However in laboratory experiments, similar increases were followed quickly by drastic reduction in the population (Filonow and Chun, 1981). Zakaria and Lockwood (1980) observed increased Fusarium populations in field soil amended with linseed and cottonseed meals, and suggested that insufficient soil moisture, incomplete incorporation of soybean meal, or escape of volatile degradation products might have been responsible. However, at Rose lake, particular attention was paid to avoid these possibilities. The Rose Lake trial began in early fall, and low soil temperatures may have delayed the rate of breakdown of soybean meal or reduced the effectiveness of any volatile produced. Papavizas et al. (1970) observed that soybean meal was ineffective in reducing bean root rot by T. basicola at 16°C but was effective at 22, 28, and 35°C. Loss through volatilization may not be an important factor since the results in covered and uncovered plots did not differ appreciably.

Urea, applied at 1% of soil weight, was effective in reducing the number of viable propagules of all three pathogens in all three microplot trials. Pythium ultimum was the most sensitive of the three pathogens, responding the most rapidly and to the lower concentrations

of urea; T. basicola and M. phaseolina followed in decreasing order of sensitivity. The populations of M. phaseolina in trials 1 and 2 showed a tendency to increase slightly in the untreated and treated soils during some sampling periods. Whether this was due to saprophytic growth of the pathogen (Bhattacharya and Samaddar, 1976) or to fragmentation of the sclerotial pieces was not determined. Urea at 0.1% reduced the populations of the three pathogens in trial 1, but not in trial 2. The 0.25% urea treatment reduced levels of all three pathogens in trial 3.

The failure of 0.1% urea to reduce pathogen populations and the slower decline in populations at 1.0% in trial 2 was probably because this experiment was begun in early fall, when low soil temperatures may have reduced the toxicity of ammonia. Similarly, the failure of soybean meal to reduce P. ultimum populations in the field may also have been due to low soil temperature since this experiment was also done in the fall. These results are consistent with the observation of Papavizas et al. (1970) that bean root rot caused by Thielaviopsis basicola was reduced in soil that had been amended with soybean meal at 35, 28, and 22 C but not at 16 C.

The reduction in pathogen populations by urea amendment of soil is attributed to the formation of ammonia. Urea is readily degraded by soil microorganisms into ammonia and carbon dioxide, and ammonia generated by hydrolysis of urea was toxic to the sclerotia of M. phaseolina in vitro. Ammonia was detected in trials 2 and 3 in proportion to the amount of urea applied. Ammonia was not monitored in trial 1, but its odor was apparent. Ammonia has been implicated in the

reduction in propagule numbers of Phytophthora cinnamomi by urea (Tsao and Zentmyer, 1979), and of Rhizoctonia solani by plant residues of low C:N ratio (Lewis and Papavizas, 1975). Anhydrous ammonia is toxic to a number of fungi (Rush, 1982; Rush and Lyda, 1982; Smiley et al., 1972).

The mode of action of ammonia in reducing pathogen populations in soil is uncertain. Reductions in populations of Phytophthora cinnamomi and Phytophthora parasitica and suppression of disease development of avocado seedlings in urea-treated soils were associated with inhibition of germination and formation of sporangia in soil extracts, suggesting a direct toxic effect of the NH_3/NH_4^+ and NO_2^-/HNO_2 from the breakdown of urea (Tsao and Zentmyer, 1979). In other work in our laboratory ammonia stimulated propagule exudation in vitro and this was associated with death of the propagules (Chun et al., 1984; Filonow et al., 1981). Moreover, exposure to concentrations of ammonia which were non-lethal during shorter exposures, reduced longevity of Cochliobolus sativus upon longer incubation. Increased soil pH was associated with ammonia treatments, but did not account for the greater exudation effected by ammonia, nor for its lethal effects (Chun et al., 1984). In our field experiments, prolonged, increased exudation from ammonia may have contributed to the decline in populations of the root-infecting pathogens. Decreased germinability of fungal propagules has been associated with increased exudation occurring during incubation in soil (Filonow et al., 1983). Ammonia may bave been involved in the long-term decline in populations of M. phaseolina sclerotia occurring in nitrogen-amended soil in sealed containers (Filho and Dhingra, 1980).

Although urea effectively reduced populations of P. ultimum, T. basicola, and M. phaseolina in our experiments, the application of excess nitrogen could be detrimental to crops, and its widespread use may be prohibitively expensive. At the current price of $ .248/kg of urea, the approximate cost would be $562-703 per acre ($1406-1758 per hectare) to treat a field at the 0.25% rate to 15.2-17.8 cm depth. Further research might focus on urea application in localized areas of pathogen infestation or where low concentrations can be applied that might bring about a gradual population decline, and on possible unfavorable effects of excess nitrogen to field crops. Our results indicate that the lowest effective urea concentration is between 0.1% and 0.25%, the choice being dependent on the pathogen, the soil temperature, and how quickly the population is to be reduced.

Chapter 5

IMPROVEMENTS IN ASSAYS FOR SOIL POPULATIONS OF *PYTHIUM ULTIMUM* AND *MACROPHOMINA PHASEOLINA*

5.1 INTRODUCTION

Pythium ultimum Trow. and *Macrophomina phaseolina* (Tassi) Goid. [=*M. phaseoli* (Maubl.) Ashby] are soil-borne pathogens of many plants throughout the world. *P. ultimum* is responsible for pre- and post-emergence damping-off and *M. phaseolina* causes charcoal rot of corn, soybean, and other crops in arid and semi-arid soils. Many techniques have been developed to enumerate these pathogens. Principle among these have been selective media (Tsao, 1970) and a soil-particle technique (Schmitthenner, 1962) for selective enumeration of *Pythium*. Stanghellini and Hancock (1970) developed a rapid and accurate method for the direct isolation of *P. ultimum* from soil based on its rapid propagule germination and fast growth rate. Aliquots of the soil dilutions were spotted on the surface of agar plates and the hyphae from the germinating sporangia were counted. For *M. phaseolina*, sclerotia usually are recovered from soil by wet and/or dry sieving and the retained material is plated onto a selective medium and the colonies counted (McCain and Smith, 1972; Meyer et al., 1973; Mihail

and Alcorn, 1982; Papavizas and Klag, 1975). Watanabe et al. (1970) recovered sclerotia from soil by differential flotation and were able to identify and determine viability of the pathogen by culturing on potato-dextrose agar (PDA).

The large number of soil samples generated from field experiments in which the populations of the above two pathogens and Thielaviopsis basicola were monitored simultaneously over time (Chun and Lockwood, 1982a) could not be handled by the above methods and led to improvements and modification to some of the above methods. Specifically, a 'well' plate method, modified from that of Stanghellini and Hancock (1970), was developed to assay P. ultimum and a flotation method, employing a procedure used to isolate microsclerotia of Verticillium albo-atrum from soil (Huisman and Ashworth, 1974), was used to separate sclerotia of M. phaseolina from soil before plating them on a selective medium. A preliminary report has been published (Chun and Lockwood 1982b).

5.2 MATERIALS AND METHODS

5.2.1 Culture of pathogens and preparation of infested soil.

P. ultimum was obtained from soybean seedlings (Schlub and Lockwood, 1981), and was maintained on carrot agar (Chun et al., 1984). M. phaseolina isolate No. 4 was obtained from T. D. Wyllie, University of Missouri, and was maintained on PDA.

Sporangia of P. ultimum and sclerotia of M. phaseolina were used to infest soil. P. ultimum was cultured in 2 L of 0.25 or 0.1 strength carrot broth in autoclavable bags (Bio-Check Biohazard bags, American Scientific Products, McGaw Park, Il. 60085) at 24-26°C for 2-3 weeks. The mycelial mats of P. ultimum were rinsed with distilled H_2O, homogenized in an Omni-mixer (Ivan Sorvall, Norwalk, CT 06856), and manually mixed into sandy loam field soil in the laboratory. The soil was kept moist at 24-26°C for at least a week to permit lysis of mycelial fragments, then was air-dried, and stored at 4°C. M. phaseolina was cultured in 500 ml of PD-broth in aluminum pans (50 x 29 x 8 cm) covered with aluminum foil, or in 80 ml of broth in Roux bottles at 24-26°C for 2-3 weeks. Mycelial mats of M. phaseolina were rinsed with distilled H_2O, dried with forced air, then crushed manually. The sclerotia were passed through a 177 µm screen to partially separate the individual sclerotia. The sclerotia were stored at 24-26°C and added to air-dried soil as needed.

5.2.2 Assay for M. phaseolina.

To recover sclerotia of M. phaseolina from soil, 60% (w/w) sucrose (commercial table sugar) solution was added to the 40 ml mark of 50 ml screw capped polypropylene centrifuge tubes, each containing 5-10 g of wet or dry infested soil. Tubes were placed on a reciprocal shaker for 1 h, after which additional sucrose was added to the tubes bringing the sucrose level to the 50 ml mark. The tubes were centrifuged for 10 min at 3100 X g and the sclerotia were aspirated off the top of the sucrose solution, the centrifuge cap, and the wall and lip of the tube, and

collected on nylon 15 µm screens fastened to collection tubes. A stream of distilled water from a wash bottle was used to loosen sclerotia adhering to the walls of the centrifuge tube and cap during collection.

The collection tubes consisted of 1.0 ml tuberculin syringe barrels with the basal flange cut off. Nylon screens (4 cm^2, 15 µm openings) (Tetko, Inc., Elmsford, N. Y. 10523) were secured over the large open ends by plastic bands (Tygon). The screen ends were attached to a vacuum source via 0.64 cm diam. Tygon tubing. Sclerotia on the nylon mesh were rinsed free of sucrose by aspiration with 100 ml of distilled H_2O, then were surface-sterilized by dipping the collection tubes in 0.5% NaOCl solution for 5 min followed by sterile rinse water. For multiple samples, collection tubes were bound together with rubber bands and treated as a unit for surface-sterilization and rinsing. Before removing the screens, a small quantity of sterile water was drawn into the tubes to pull sclerotia adhering to the inner barrel onto the screen. Screens were placed in 10 ml aliquots of 0.2% water agar to suspend sclerotia. Selective medium (Papavizas and Klag, 1975) was poured into dishes containing variable aliquots of the sclerotial suspension, and the colonies were counted after 7-10 days incubation at 30°C in the dark.

Many soil types, including dried clay loam and either dry or moist soil collected directly from the field, was observed to disperse readily in 0.2% water agar or in 60% sucrose by shaking on a reciprocal shaker for 1 h. Fungal propagules remained uniformly suspended in 0.2% water agar; in particular, sclerotia were observed to remain suspended

for several days whereas sclerotia in water, saline, or buffered media settled after a few minutes.

Efficiency of recovery was determined by adding 4.0 mg of sclerotia to 5.0 g of soil and enumerating them using the above method. This was compared with the number of colonies formed when 4.0 mg of sclerotia was surface-sterilized in 0.5% NaOCl as above but for 1 minute, then plated on the selective medium. Four replicates (1 or 3 plates/replicate) were used.

5.2.3 Assay for P. ultimum.

Initial 1:10 (w/v) soil dilutions were made in 0.2% water agar and shaken on a reciprocal shaker for 1 h. A total of 1.0 ml of the dilution was dispensed into 50-60 wells (2.5 mm diam.) (about 0.01 ml per well) made in 2% water agar in each of two, 90 x 15 mm plastic petri dishes (13 ml agar/plate). To apply the soil dilutions to the agar wells small dispensing pipettes were made from 2.5 or 3.0 cc plastic syringes, each attached with Tygon tubing to a disposable 14.7 cm glass pasteur pipette. Each unit was calibrated to draw 1 ml. A tiny hole made above this mark allowed the soil dilution to be released slowly from the pipette tip when the plunger was withdrawn beyond the hole. By adjusting the angle, the flow rate from the syringe could be controlled to facilitate filling the wells. The agar wells were spaced 1 cm apart and were made by a small brass tube connected to a vacuum line. The dishes were inverted and incubated at 24-26°C for 18-24 h. The number of hyphal strands emerging from the walls of the wells in inverted plates were counted under a dissecting microscope at 180 x

magnification using transmitted fluorescent lighting.

Since the number of sporangia remaining viable after incorporation into soil could not be determined, the efficiency of recovery remained unknown. Instead, the assay to quantify P. ultimum in soil was compared with that of Stanghellini and Hancock (1970). Since suspending the soil on the reciprocal shaker for 1 h favored the 'well method', soil dilutions of the P. ultimum infested soil were made in 0.2% water agar and mixed for 2 min using a vortex mixer. One ml aliquots were removed and alternately assayed by the method of Stanghellini and Hancock (1970) where aliquots of the soil dilutions were spotted on the surface of agar plates and the hyphae from the germinating sporangia were counted emerging from the spot, or by the 'well method' where the aliquots were dispersed in cylindrical wells and the hyphae emerging from the cylindrical wall were counted. Eight replicates were done and the experiment was repeated.

5.3 RESULTS AND DISCUSSION

Our procedure recovered 97.5% and 95.6% of added sclerotia from soil in two experiments. All the materials used are readily available, making it possible to process many soil samples in a batch, if desired. Mihail and Alcorn (1982) pointed out that the selective medium of Papavizas and Klag (1975) permitted growth of other organisms. However, this was not a problem in our procedure, since contaminating soil was removed and the sclerotia were surface-

sterilized. This selective medium was chosen because M. phaseolina grew as small easily discernible colonies. Non-viable sclerotia could be differentiated from viable sclerotia if the plates were examined earlier and if greater dilutions were used. M. phaseolina population assay can be varied to accommodate different amounts of soil and the anticipated recoverable number of viable sclerotia. Recovery of sclerotia by flotation on a sucrose solution coupled with the collection and surface-sterilization procedures described permitted a large number of samples to be processed easily and rapidly, whereas the currently used procedures require drying the soil, then crushing and dry sieving (Meyer et al., 1973; Mihail and Alcorn, 1982), and/or blending the soil (Mihail and Alcorn, 1982; Papavizas and Klag, 1975) followed by wet sieving steps before plating on selective medium.

The method of Stanghellini and Hancock (1970) to enumerate P. ultimum is simple and rapid to use. However, in our experience in field (Chun and Lockwood, 1982) and laboratory trials (Filonow and Chun, 1981), the fungus occasionally branched early after germination which could lead to erroneous population estimations. A wide variation in populations of P. ultimum were observed in field trials (Chun and Lockwood, 1982) and so a range of soil dilutions was used. The lower dilutions had more soil particles which obscured hyphal branching within the sites of inoculation and made the plates difficult to read. One way to circumvent this was to use smaller drops on the plates and to disperse a given dilution over a larger number of plates, but this proved to be uneconomical. The well method was developed to reduce the number of plates used. It was useful over a wider range of populations

and was more easily read than the original method (Stanghellini and Hancock, 1970) at low soil dilutions. Preliminary tests suggested that population counts were linear with soil dilutions. Earlier comparisons of the two methods suggested that the well method was more sensitive, which may have resulted from better soil dispersal using the reciprocal shaker for 1 h and the 0.2% water agar suspending medium. Even when samples were suspended in the same manner, the well method gave consistently higher counts (32.1±1.8 vs. 20.8±1.4 ; 36.2±1.9 vs. 32.6±3.6), but the differences were not always statistically significantly. The well method appears to offer some advantages over the original method in that it uses fewer petri plates, was slightly more sensitive, afforded more latitude in the incubation time, and required less time to perform. Where low population numbers require low soil dilutions, the well method also provided greater visibility and therefore more accurate counts.

Chapter 6

INTERACTIVE EFFECT OF AMMONIA AND PH ON VIABILITY AND ^{14}C-LABELED EXUDATION FROM FUNGAL PROPAGULES

6.1 INTRODUCTION

The toxicity of ammonia to fungi is well known (Eno et al., 1955; Gilpatrick, 1969a, 1969b; Leach and Davey, 1935; Neal and Collins, 1936; Rush and Lyda, 1982; Tsao and Zentmyer, 1979). Efforts to capitalize on this toxicity for the control of soil-borne plant pathogenic fungi have included the application to soil of anhydrous ammonia (Eno et al., 1955; Rush, 1981; Smiley et al., 1970), high nitrogen-containing plant residues (Gilpatrick, 1969a, 1969b), animal manures (Tsao and Oster, 1981), ammonium salts (Filho and Dhingra, 1980; Henis and Chet, 1968), and urea (Chun and Lockwood, 1982; Tsao and Zentmyer, 1979).

Ammonia is also one of several volatile compounds implicated as a cause of fungistasis (Lockwood and Filonow, 1981), particularly in alkaline soils (Ko and Hora, 1974; Ko et al., 1974; Pavlica et al., 1978), in soils made alkaline by the addition of lime (Hora and Baker, 1974; Pavlica et al., 1978), or in soil treated with chitin (Schippers and Palm, 1973). How ammonia operates as a toxic or fungistatic agent

has not been determined.

Nutrient deprivation is an alternate causal mechanism proposed for fungistasis (Lockwood and Filonow, 1981). Considerable evidence has accrued which shows that the germination of fungal propagules which do not require exogenous nutrients for germination (nutrient-independent propagules) can be repressed by incubation in a model apparatus simulating soil-imposed nutrient stress (Filonow and Lockwood, 1979; Lockwood and Filonow, 1981). Enhanced exudation of ^{14}C-labeled compounds from nutrient-independent propagules has been associated with the imposition of soil fungistasis (Filonow and Lockwood, 1983a; 1983b; Lockwood and Filonow, 1981). Prolonged exposure of fungal propagules to nutrient stress can debilitate them, resulting in elevation of their nutrient requirements for germination, in a progressive loss of viability (Bhattacharya and Samaddar, 1976; Filonow and Lockwood, 1983b), and in decreased virulence (Filonow et al., 1983).

Since fungi in soil may be subjected to both nutrient stress and chemical inhibitors, it was of interest to determine if there was a relationship between a chemical stress, such as ammonia, and fungal exudation in soil. Specifically, studies were done with soil or with a model apparatus which simulates soil-imposed nutrient stress to determine whether ammonia could increase exudation from fungal propagules under nutrient stress and to determine the effects of enhanced exudation on propagule viability. A preliminary report of this work has been published (Filonow et al., 1981).

6.2 MATERIALS AND METHODS

6.2.1 Fungi.

Cochliobolus victoriae Nelson [=Bipolaris victoriae (Meehan and Murphy) Shoem.] isolate No. 3 was obtained from R. P. Scheffer, Michigan State University, and C. sativus (Ito and Kurik.) Drechs. ex Dast. [=Bipolaris sorokiniana (Sacc.) Shoem.] isolate 91, T_2T_1, was obtained from R. D. Tinline, Research Station, Agriculture Canada, Saskatoon, Saskatchewan. Macrophomina phaseolina (Tassi) Goid. [=M. phaseoli (Maubl.) Ashby] isolate No. 4 was obtained from T. D. Wyllie, University of Missouri. The fungi were grown in the dark at 24-26°C on agar media supplemented with 10 uCi (1 Ci = 37 GBq) of [^{14}C]-glucose (specific activity, 180-240 mmol/mCi). Macrophomina phaseolina was grown on potato dextrose agar (PDA) for 1 month, and C. victoriae and C. sativus were grown on carrot agar for 1-3 months. P. ultimum was isolated from soybean seedling pieces (Schlub and Lockwood, 1981) and grown on 0.1 strength carrot broth for 12-15 days at 24-26°C. Carrot agar was prepared by autoclaving 30 g of sliced carrots in 250 mL of distilled water and decanting the supernatant solution into 20 g of agar in 750 mL of distilled water. Conidia of C. victoriae and C. sativus and sclerotia of M. phaseolina were aseptically dislodged into a small volume of cold water and filtered through a 0.153- or 0.243-mm mesh nylon screen into centrifuge tubes. Propagules were washed three times by centrifugation at 5900 X g at 5°C for 5 min, resuspended in

cold distilled water, and deposited by suction on 25 mm diameter membrane filters (0.4 µm pore diameter; Nuclepore Corp., Pleasanton, CA). Propagule density on filters was adjusted to give between 71,000 to 108,000 disintegrations per minute (dpm) (ca. 40,000-60,000 conidia of C. victoriae per filter or 200-400 sclerotia of M. phaseolina per filter). In other experiments propagules adjusted to give 1,800 dpm were deposited on 1 cm^2 membrane filters. The filters were placed in scintillation vials, dissolved in 0.3-0.6 mL of $CHCl_3$, and suspended in 10 mL Cab-O-Sil scintillation cocktail to determine radioactivity. Scintillation counts were corrected for counting efficiency by using a $[^{14}]$C-toluene internal standard. Preparation of scintillation cocktails and determination of activity have been described (Filonow and Lockwood, 1979; 1983a) .

Mycelial mats of P. ultimum were collected on 0.08-mm mesh nylon screens, rinsed with water, and then homogenized with an Omni-mixer (Ivan Sorvall, Norwalk, CT 06856) at 10,400 rpm for 1 minute. The homogenate was first passed through a 0.08-mm and a 0.035-mm mesh nylon screens and then through 0.015- or 0.02-mm mesh nylon screens to collect the sporangia of P. ultimum (remaining mycelial fragments were found in previous tests to be nonviable). The sporangia were rinsed with water and then suspended in 0.2% water agar for use.

6.2.2 Effect of ammonia on ^{14}C-labeled exudation from labeled propagules incubated on urea-supplemented soil.

Determination of ^{14}C-labeled exudation from labeled propagules on soil was made in a manner similar to that of Filonow and Lockwood

(Filonow and Lockwood, 1979; 1983a). A sandy loam soil was sieved (42 mm mesh) and kept at about -0.1 bars soil matric potential (1 bar = 100 kPa) for 3 or more days at 24-26°C before use. Urea granules (Mallinckrodt, Inc., Paris, KY) were ground to a coarse powder and mixed with the soil to give 1 and 5% (w/w) urea-soil mixtures. Soil without urea was the control. Immediately, 10 or 20 g of the soil mixture was transferred to 60 mL polypropylene incubation chambers (Filonow and Lockwood, 1979), and the soils were wetted to saturation. Four chambers per treatment were used. A filter bearing ^{14}C-labeled propagules was then placed on the soil and the chambers were attached to a $^{14}CO_2$ trapping apparatus (Filonow and Lockwood, 1983a). After 22-24 h, scintillation vials containing $^{14}CO_2$ were collected, and the filters were removed. Viability of the propagules was determined by placing a small sample of the propagules from each filter on PDA and counting germination after an appropriate length of time (12-48 h). The radioactivity of the filters was determined as above. After the filters were removed, the soils in the chambers were frozen until the soils could be oxidized to determine residual, nonrespired ^{14}C (Filonow and Lockwood, 1979; 1983a).

The effect of ammonia from urea on soil respiration in glucose-supplemented soil was determined by adding 0.1 mL of [^{14}C]-glucose (8.5 X 10^4 dpm) to chambers containing 10 g of soil mixed with urea [0, 1, and 5% (w/w)]. $^{14}CO_2$ was collected for 4, 8, 16, and 24 h as above. pH changes were determined in soil supplemented with unlabeled glucose (1.2 mg glucose per chamber) after 4, 8, 16, 24, and 48 h. For pH determination, small samples of soil were mixed [1:2 (w/v)] with 0.01 M

$CaCl_2$.

6.2.3 Effect of ammonia and pH on ^{14}C-labeled exudation from labeled propagules incubated on leached sand.

A leached sand apparatus described for simulating nutrient stress imposed by soil was used (Filonow and Lockwood, 1983b). Sterile solutions with or without ammonia were pumped from a reservoir into an aseptic glass or polycarbonate plastic dish containing sand on which ^{14}C-labeled propagules, borne on membrane filters, were placed. The leachings, collected via an outlet in the dish, contained ^{14}C-labeled exudate from the propagules. Ammonium hydroxide (29.6% NH_3 by weight; Mallinckrodt, Inc., Paris, KY) was diluted with 0.9% (w/v) NaCl to give concentrations of 100, 1000, and 10,000 mg NH_3/L. The respective pH's of these solutions as collected were 9.6, 10.3, and 11.1. The pH of the NaCl solution was 6.5.

In most experiments propagules were used immediately after they were collected from agar and washed. Conidia of C. victoriae and sclerotia of M. phaseolina were nutrient-independent, showing > 80-90% germination on a sterile, dilute salts solution (Filonow and Lockwood, 1983a; 1983b). In other experiments, washed ^{14}C-labeled conidia of C. victoriae were incubated on sand leached at a flow rate (75 mL/h) sufficient to repress germination for 8 days, to make them nutrient-dependent. The conidia germinated about 30% on dilute salts solution (Filonow and Lockwood, 1983b). After exudation reached a low level (about 90 min), all except the two control dishes were detached from their reservoirs and reattached to reservoirs containing a different

concentration of ammonia, containing 0, 10, 50, or 100 mg NH_3/L, percolated at a flow rate of 60 mL/min. Leachings were collected in scintillation vials at 10-min intervals. These treatments were continued for an additional 90-180 min. The pH of all samples of leachings was measured, and 10 mL of aqueous scintillation cocktail (Filonow and Lockwood, 1983a) was then added to each vial. At the end of an experiment, propagule viability and radioactivity of the filters were determined as above.

6.2.4 Effect of high pH and ammonia on ^{14}C-labeled exudation from labeled conidia of C. victoriae incubated on leached sand.

Other leached sand experiments were done to separate the effect of high pH from those of ammonia. Filters bearing conidia of C. victoriae were incubated on the leaching apparatus using 0.05 M CO_2-carbonate-bicarbonate buffer (pH 7.2) in 0.9% saline solution, at a flow rate of 60 mL/h. After 90 min, the leaching solutions were changed on some of the dishes: duplicate dishes were percolated with a saline solution of 0.05 M Na carbonate - bicarbonate buffer (i) at pH 10.0; (ii) at pH 10.8; (iii) with 100 mg NH_3/L, at pH 10.0; and (iv) with 1000 mg NH_3/L, at pH 10.7. After about 70 min more, the treatments were switched so that the dishes percolated with the pH-10.8 solutions were percolated with the pH-10.7 solution with ammonia and the dishes percolated with the pH-10 solution were percolated with the pH 10 solution with ammonia and vice versa.

6.2.5 Effect of short term exposure to low levels of ammonia and alkaline conditions on survival of sporangia of P. ultimum.

The effect of alkalinity and ammonia on the viability of P. ultimum sporangia was examined by placing sporangia in solutions of 0.9% NaCl in 0.2% agar with 0.05 M Na carbonate - bicarbonate buffer or in saline solutions in 0.2% agar made alkaline by the addition of ammonia. The pH of the buffered solutions were 9.8, 10.4, and 10.7. The ammonia solutions were made up as 0, 50, and 100 mg NH_3/L. Samples were removed at intervals, diluted in 0.2% agar in 0.1 M phosphate buffer (pH 6.5) and assayed for viability using the 'well method', described elsewhere.

6.2.6 Effect of long term exposure to low levels of ammonia and alkaline conditions on survival of propagules.

Filters bearing conidia of C. sativus were placed in duplicate dishes of the leaching apparatus operated at a flow rate of 60 mL/h with 0, 10, 50, and 100 mg NH_3/L (in 0.9% saline) for 9 days. The respective pH of each solution was 6.6, 9.5, 10.2, and 10.5.

The effect of alkalinity and ammonia on the viability of M. phaseolina sclerotia was examined by placing sclerotia in solutions of 0.9% NaCl in 0.2% agar made alkaline by addition of NaOH or NH_3. The pH's of the solutions without NH_3 were 5.2 (control), 11.2, and 11.9. The pH's of the solutions with NH_3 were 10.0, 10.7, and 10.8, respectively, for 100, 500, and 1000 mg NH_3/L. At intervals, aliquots

of the sclerotial suspension were removed and plated on a selective medium (Papavizas and Klag, 1975) to determine viability.

6.2.7 Effect of high pH and ammonia on ^{14}C-labeled exudation from conidia not under nutrient stress in a static system.

Two ml of filter-sterilized 0.1 M CO_2-carbonate-bicarbonate buffer (pH 7.0, 9.0, 10.0, and 11.0), with or without NH_4Cl, were placed in sterile 22 mL screw-top vials. Appropriate amounts of NH_4Cl were added to each pH series to give concentrations of 10, 50, 100, 250, 500, 750, and 1000 mg NH_3/L of solution. As a check against possible salt effects, NH_4Cl in amounts equal to that in the pH-9.0 series was added to the pH-7.0 series. ^{14}C-labeled conidia of C. sativus on 1 cm^2 filters were floated on the solutions. After 4 h, the filters were removed and radioactivity was determined and 15 mL of aqueous scintillation cocktail (Safety-Solve, Research Products, Inc., Il) was added to the vials to determine the radioactivity in the solution. Five replicates per treatment were used. A parallel experiment with two to five replicates was run to test viability of the conidia.

The pH's were determined with glass pH electrodes (Sargent-Welch, S-30070-10; Corning, 476223) and the corrections for Na^+ effects at high pH were not made. Experiments were done at least twice with similar results.

6.3 RESULTS

6.3.1 ^{14}C loss from propagules incubated on urea-supplemented soil.

Conidia of C. victoriae incubated for 24 h on nontreated soil lost 4.2% of their ^{14}C in exudate, whereas those incubated on soil treated with 1 and 5% urea lost 10.5 and 5.4%, respectively (Fig. 13). Of the total amound exuded, 84, 66, and 42%, respectively, was accounted for by exudate respired by the soil microflora.

^{14}C lost from sclerotia of M. phaseolina after 24 h increased as the concentration of urea in soil increased (Fig. 13). Exudation on soil treated with 0, 1, and 5% urea was 1.7, 3.8, and 5.0% of total ^{14}C, respectively. Corresponding values for that proportion collected via respired ^{14}C were 41, 26, and 12% of the total ^{14}C lost from sclerotia.

Cumulative soil respiration of [^{14}C]-glucose decreased as the concentration of urea increased (Table 17 and Fig. 13) and pH of the soil amended with unlabeled glucose increased from pH 5.8 in the controls to 7.6 and 8.6 with the 1 and 5% urea after 24 h (Table 17), respectively.

Figure 13. Percent of total ^{14}C activity exuded by labeled conidia of C. victoriae and sclerotia of M. phaseolina and proportion of total ^{14}C activity respired in soil supplemented with labeled glucose and with 0, 1, and 5% urea after 24 h.

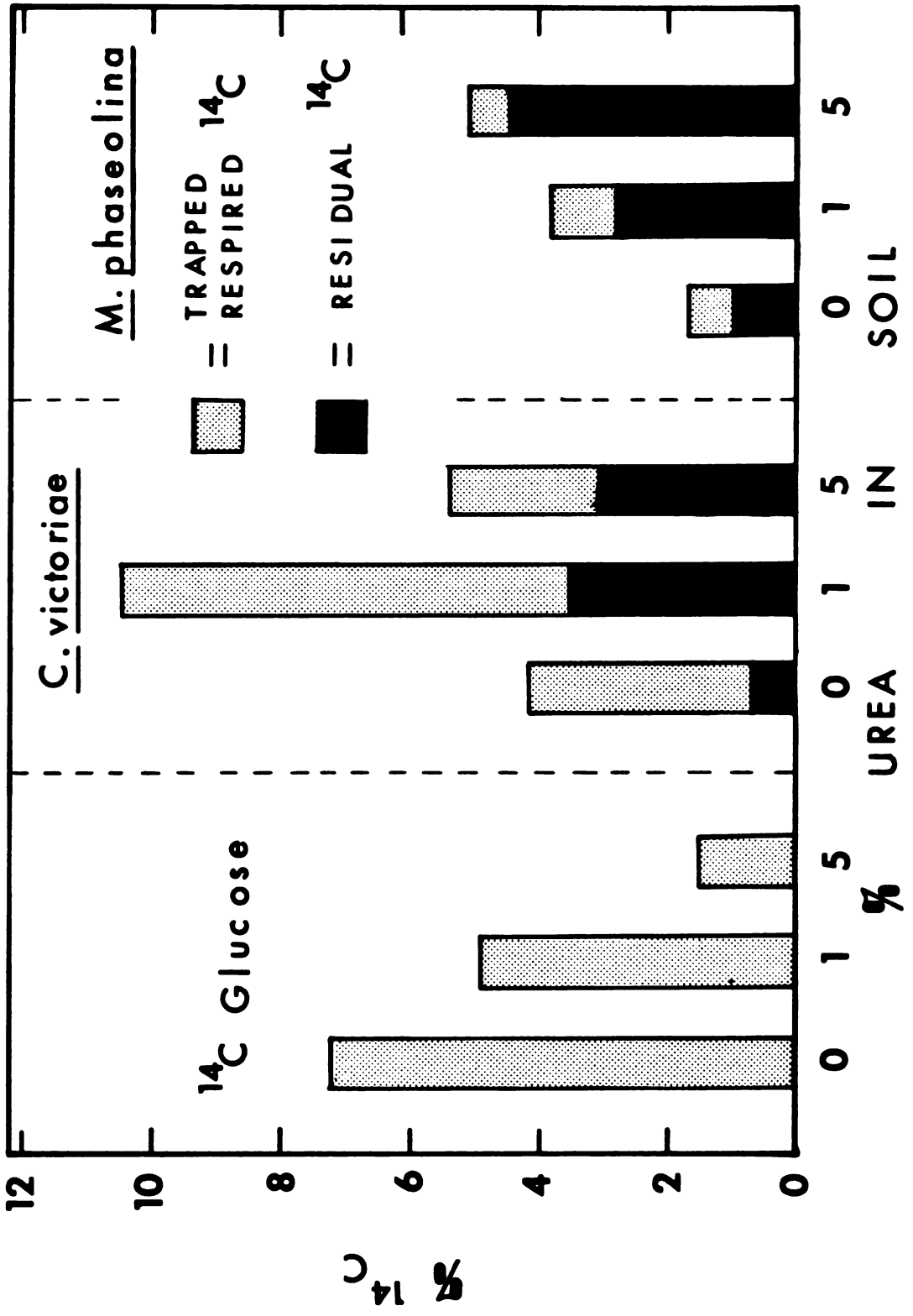

M. phaseolina
C. victoriae
^{14}C Glucose
TRAPPED = RESPIRED ^{14}C
RESIDUAL ^{14}C
0
1
5
UREA %
IN SOIL
% ^{14}C
0
2
4
6
8
10
12

TABLE 17. Cumulative respiration of [^{14}C]-glucose and pH changes in soil containing urea

	0% urea		1% urea		5% urea	
Time (h)	% respired	pH	% respired	pH	% respired	pH
0		6.0		6.0		6.1
4	3.7	5.6	2.8	6.2	1.0	6.4
8	4.5	5.5	3.5	6.6	1.4	6.9
16	5.9	5.7	4.3	7.2	1.4	7.8
24	7.3	5.8	4.9	7.6	1.6	8.6
48		5.8		8.3		9.0

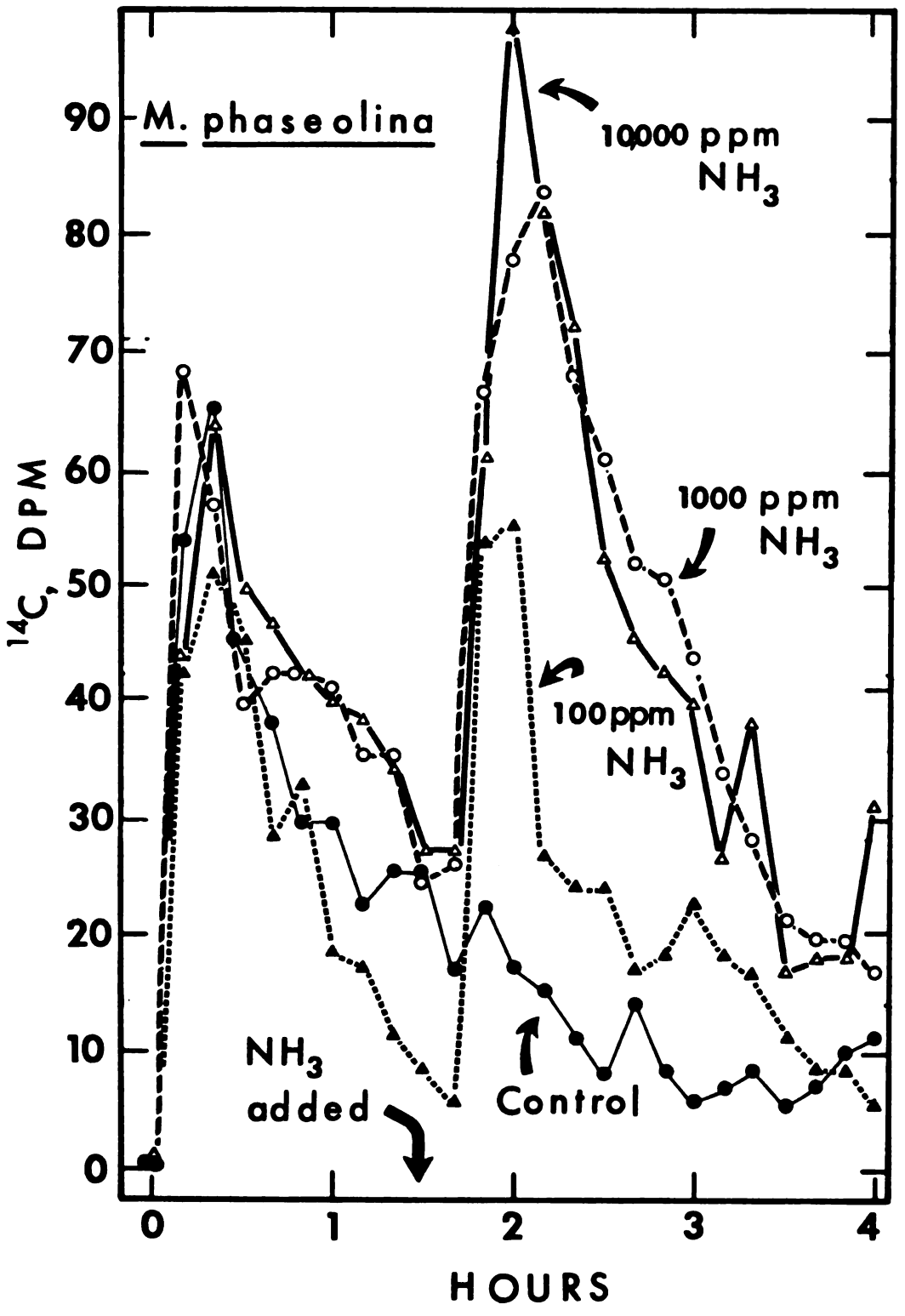
6.3.2 ^{14}C-labeled exudation from ammonia-treated propagules incubated on leached sand.

^{14}C-labeled exudation from nontreated sclerotia of M. phaseolina reached a maximum after 10 min of incubation on leached sand (Fig. 14). Subsequent incubation resulted in decreasing ^{14}C-labeled exudation until addition of ammonia, which after 20 min resulted in greatly increased ^{14}C-labeled exudation persisting for only about an

hour, even though ammonia was continually supplied. Increasing concentrations of ammonia resulted in increasing losses of ^{14}C. In a typical experiment, during 4 h incubation on leached sand, ^{14}C-labeled exudation was 0.8, 1.3, 2.1, and 2.7% of total label from sclerotia treated with 0, 100, 1000, and 10,000 mg NH_3/L, respectively. Nontreated sclerotia exuded at an erratic but lower level than ammonia-treated sclerotia. The cumulative exudate taken after addition of ammonia to the end of the experiment was two to six times greater than that for the nontreated sclerotia.

Nontreated sclerotia germinated >80% after 24 h on PDA, whereas germination of sclerotia treated with 100 or 1000 mg NH_3/L required about 36 h for a similar degree of germination. Sclerotia treated with 10,000 NH_3/L did not germinate even after 72 h.

Figure 14. Effect of ammonia from NH_4OH, at concentrations of 100, 1,000, and 10,000 mg/L (ppm), on exudation from ^{14}C-labeled sclerotia of M. phaseolina on the leaching system.

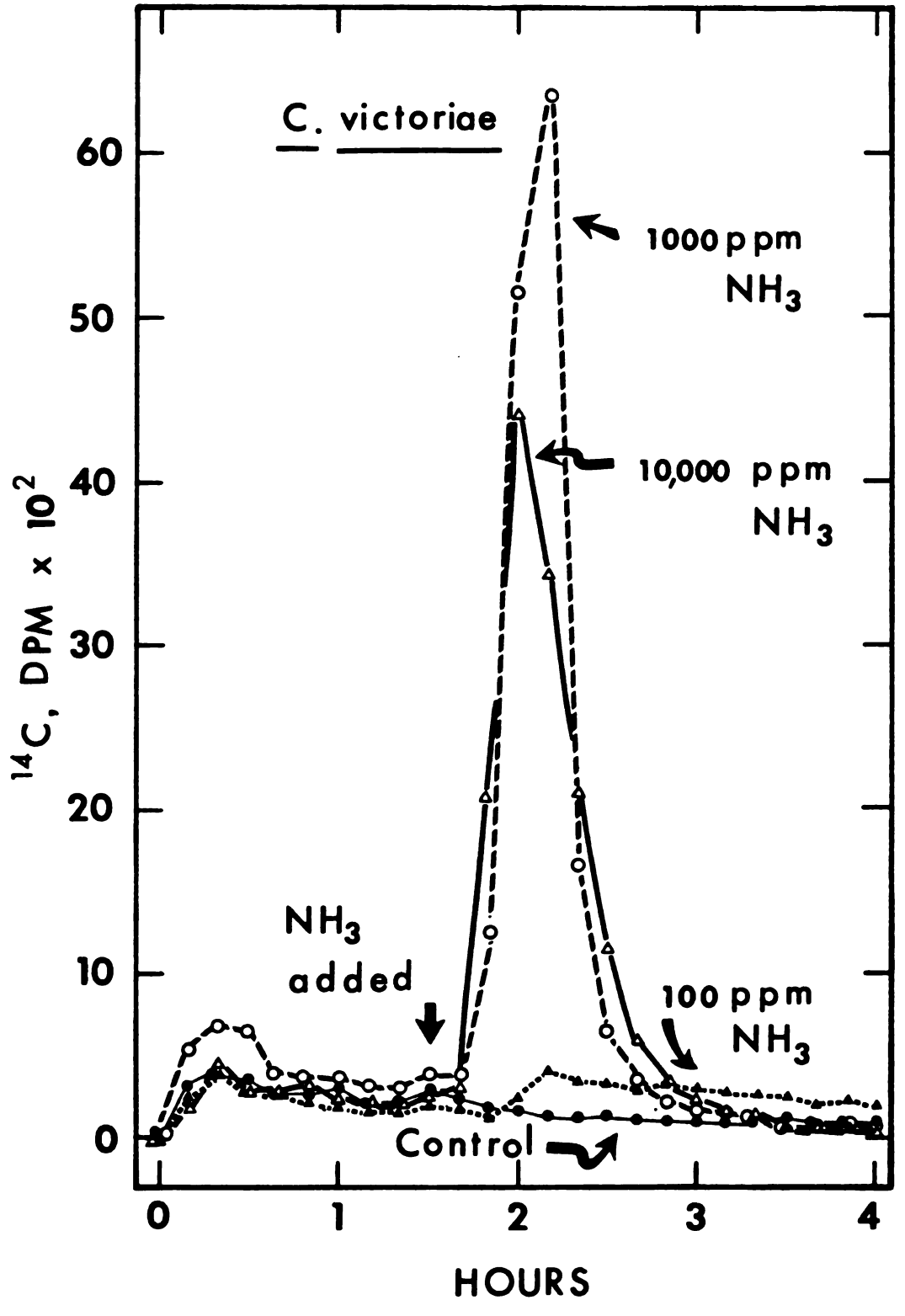

M. phaseolina
10,000 ppm NH3
1000 ppm NH3
100ppm NH3
NH3 added
Control
14C, DPM
HOURS
0
10
20
30
40
50
60
70
80
90
0
1
2
3
4

^{14}C-labeled exudation from nontreated conidia of C. victoriae also attained a maximum very quickly, and then decreased to a low level (Fig. 15). Twenty min after the addition of 1000 or 10,000 mg NH_3/L, ^{14}C-labeled exudation was sharply increased followed by a quick return in about an hour to the baseline exudation rate established prior to treatments. ^{14}C-labeled exudation from conidia treated with 100 mg NH_3/L was first observed about 40 min after application of ammonia, and was released at a much lower and gradual rate, which was about twice that of the control until the end of the experiment (4 h). Total ^{14}C-labeled exudate lost from conidia treated with 0, 100, 1000, 10,000 mg NH_3/L were 4.2%, 5.1%, 17.3%, and 13.0%; however, losses of ^{14}C from the time ammonia was introduced represent increases of two, eight, and six times, respectively, over the controls.

Nontreated conidia germinated 93% on PDA after 6 h, whereas conidia treated with 100 mg NH_3/L germinated <50% in the same period; by 24 h germination was >90%. Conidia treated with 1000 and 10,000 mg NH_3/L failed to germinate on PDA after 72 h.

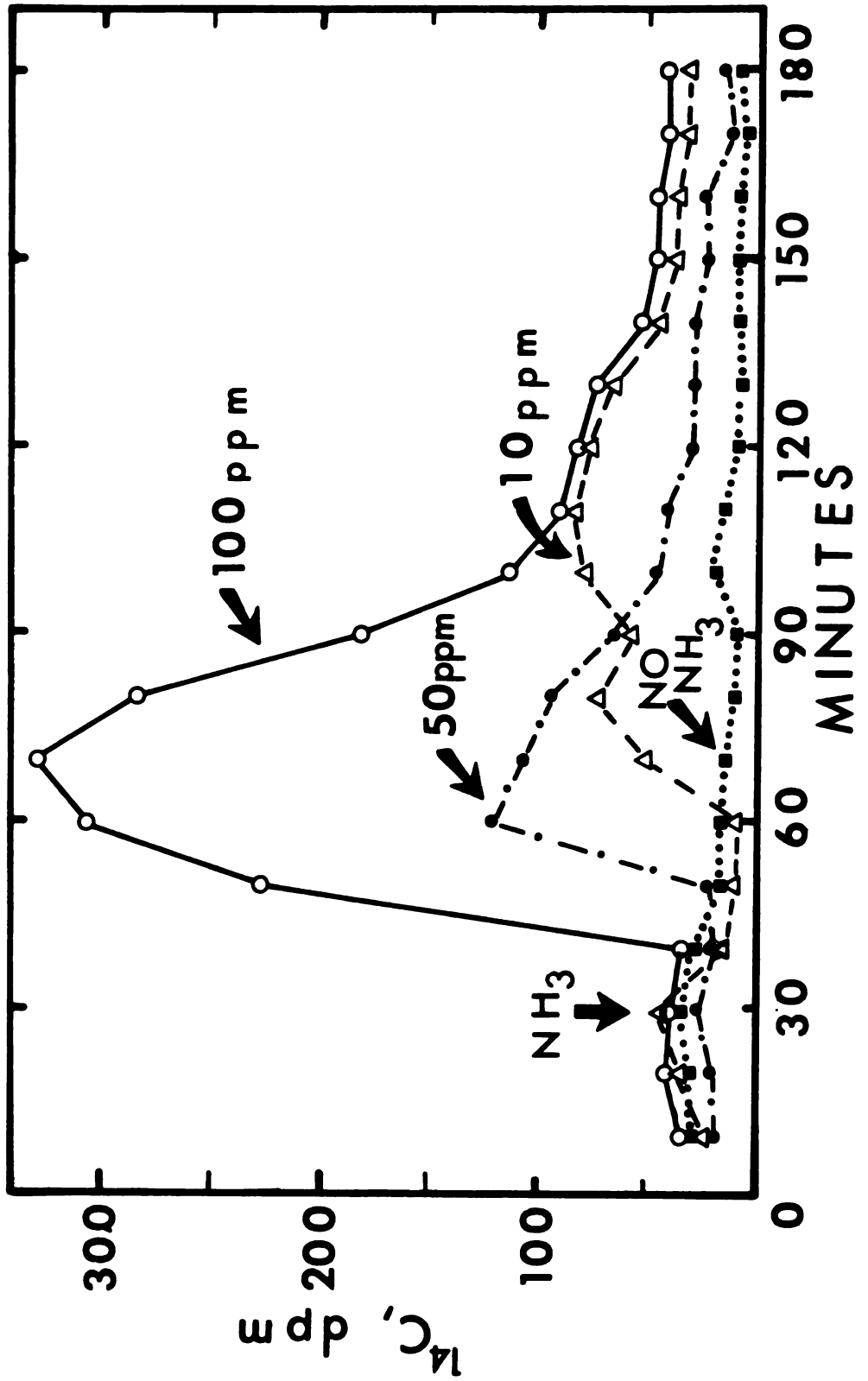
Ten minutes after the addition of ammonia to the leaching solution, pH of effluent from the sand beds increased from about 6.8 in controls to 7.4, 8.8, and 9.5 in sand percolated with 100, 1000, and 10,000 mg NH_3/L, respectively. Forty minutes after ammonia addition, pH of effluent had increased to 10.0, 10.7, and 11.4. At the end of the experiment, pH was 6.5-6.8, 9.6-10.2, 10.3-10.9, and 11.1-11.6 for sand percolated with 0, 100, 1000, and 10,000 mg NH_3/L saline solution, respectively.

Figure 15. Effect of ammonia from NH_4OH, at concentrations of 100, 1,000, and 10,000 mg/L (ppm), on exudation from ^{14}C-labeled conidia of C. victoriae on the leaching system.


C. victoriae
1000 ppm NH3
10,000 ppm NH3
100 ppm NH3
NH3 added
Control
14C, DPM x 10^2
HOURS
0
10
20
30
40
50
60
0
1
2
3
4

Nutrient-dependent conidia of C. victoriae exuded ^{14}C at a very slow rate until the addition of ammonia at 10, 50, or 100 mg/L, which stimulated exudation (Fig. 16); the increased exudation was in proportion to the ammonia concentration and the response time was more rapid as ammonia concentration increased. Viability of nutrient-dependent conidia was not decreased by ammonia at 100 mg/L when compared with nontreated conidia.

Figure 16. Effect of ammonia from NH_4OH, at concentrations of 10, 50, and 100 mg/L (ppm), on exudation from ^{14}C-labeled conidia of C. victoriae on the leaching system. The zero starting time was after 8 days of incubation on the leaching system.


^{14}C, dpm
300
200
100
0
30
60
90
120
150
180
MINUTES
100 ppm
10 ppm
50ppm
NO
NH_3
NH_3

6.3.3 Viability of P. ultimum after short term exposure to high alkaline conditions and low concentrations of ammonia.

Sporangia viability was reduced by short term exposure to either alkaline conditions or ammonia (Fig. 17). However, at the 50 or 100 mg NH_3/L level, viability reduction was much greater than observed at roughly equivalent pH's.

6.3.4 Viability of propagules exposed to low concentrations of ammonia and alkaline conditions for longer periods.

Since low concentrations of ammonia appeared to be nonlethal over a relatively short period of time, the effect of longer term exposure was studied. Cochliobolus sativus conidia remained fully viable throughout 9 days of incubation on leached sand without ammonia or in the presence of 10 mg NH_3/L (Fig. 18). However, conidia continually exposed to 100 mg NH_3/L were dead after 3 days, and those exposed to 50 mg NH_3/L were dead after 7-8 days. Cochliobolus victoriae incubated under alkaline conditions (pH 8.2-10.4), approximating those of solutions containing 50 and 100 mg NH_3/L (pH 10.2 and 10.5, respectively), did not exhibit decrease in viability after a 3-day exposure (D. Chun, unpublished data).

Figure 17. Effect of ammonia from NH_4OH, at concentrations of 0, 50, and 100 mg/L (ppm), and the effect of alkaline pH's on viability of sporangia of P. ultimum in a static system for 4 hours. CFU, colony-forming units.

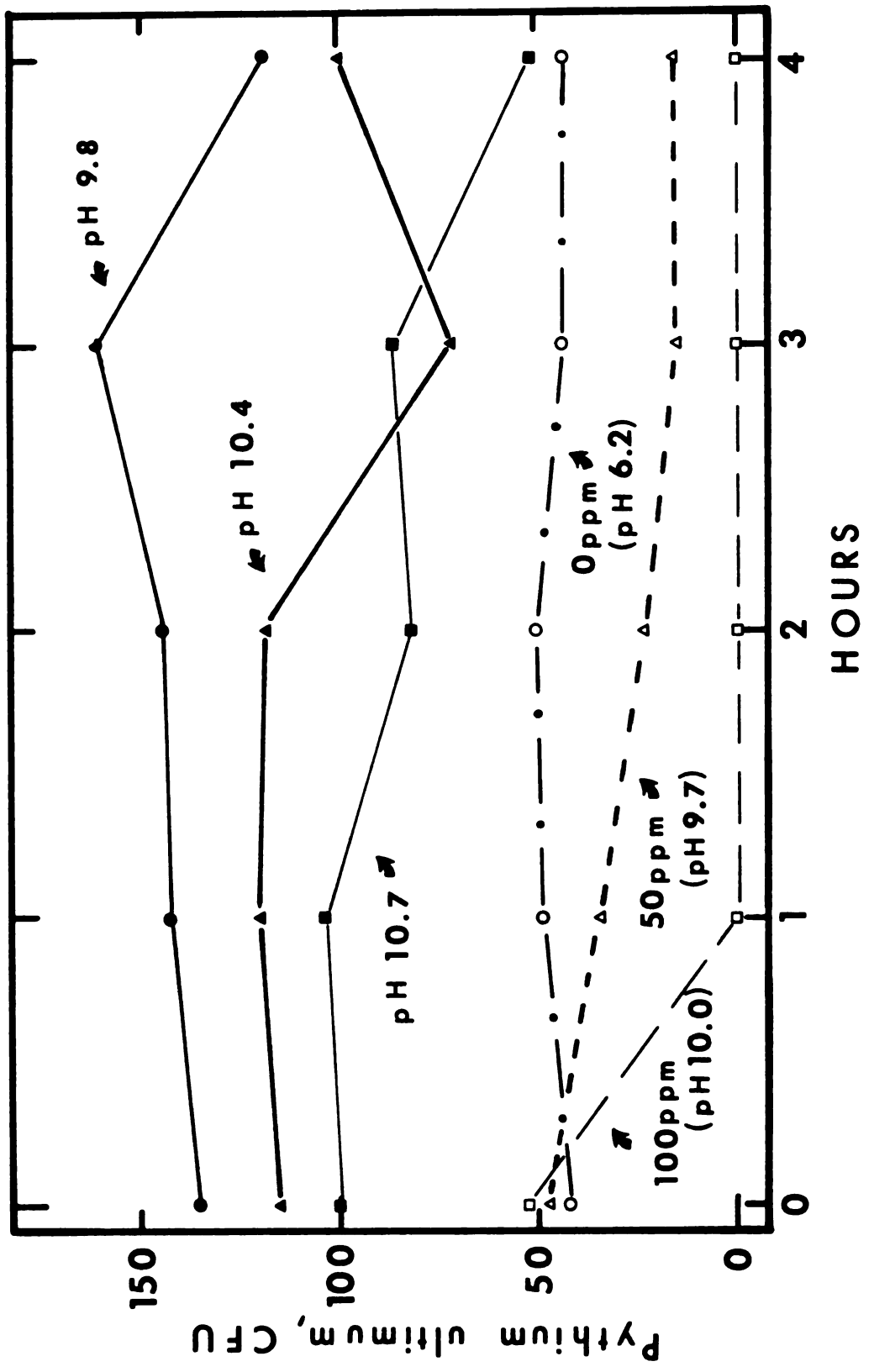
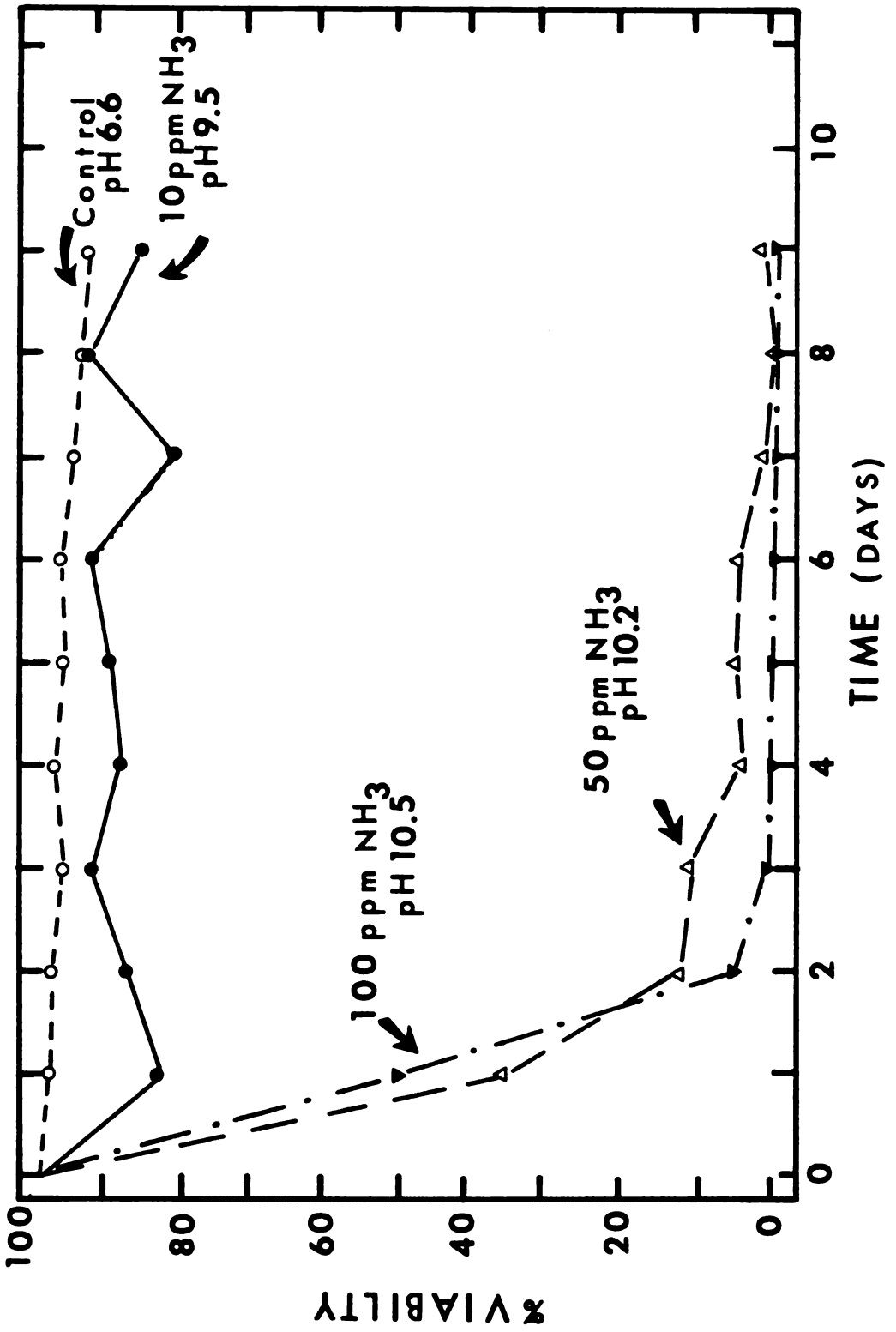
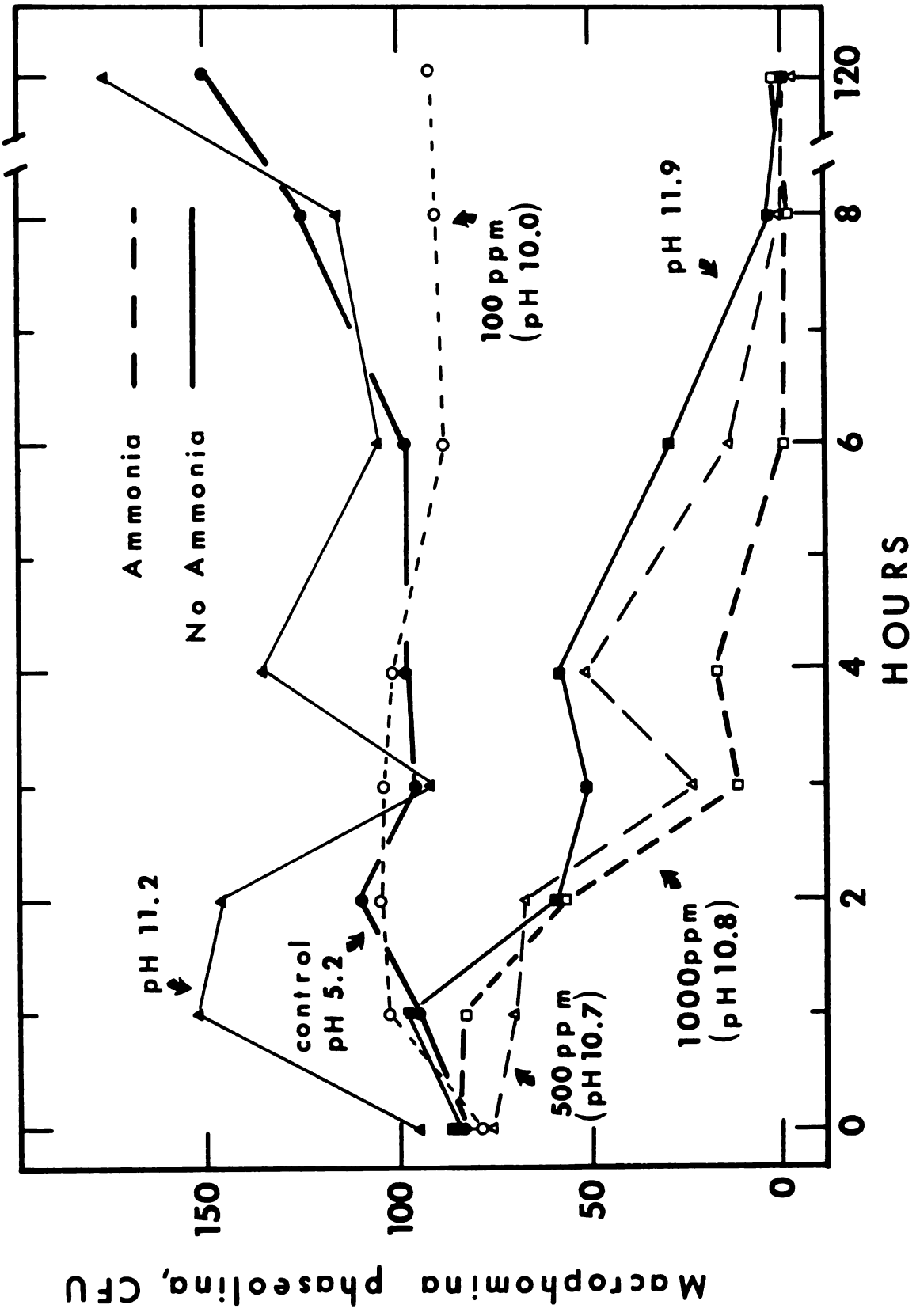

Pythium ultimum, CFU
150
100
50
0
0
1
2
3
4
HOURS
pH 9.8
pH 10.4
pH 10.7
0ppm (pH 6.2)
50ppm (pH 9.7)
100ppm (pH 10.0)

Figure 18. Effect of ammonia from NH_4OH, at concentrations of 10, 50, and 100 mg/L (ppm), on viability of conidia of C. sativus on the leaching system for 9 days.


Control
pH 6.6
10 ppm NH3
pH 9.5
100 ppm NH3
pH 10.5
50 ppm NH3
pH 10.2
% VIABILITY
TIME (DAYS)
0
20
40
60
80
100
0
2
4
6
8
10

Macrophomina phaseolina sclerotia showed much greater tolerance to ammonia than either C. victoriae or C. sativus (Fig. 19). Sclerotia were not killed until 3-6 h of exposure to 1000 mg NH^3/L. Some of the sclerotia retained viability after 6 h with 500 mg NH_3/L, but none remained alive after 8 h. By contrast, alkaline solutions with pH's comparable with those of the ammonia treatments (pH 10.7 and pH 10.8) were much less toxic. However, at pH 11.9, viability decreased at about the same rate as with the ammonia treatments. pH 11.2, which is higher than those of the solutions containing ammonia, did not affect viability through the 5-day period and in fact supported an increase in population between 6 and 120 h. A similar increase occurred with the control (pH 5.2) solutions. Ammonia at 100 mg/L neither reduced viability nor favored growth of the fungus.

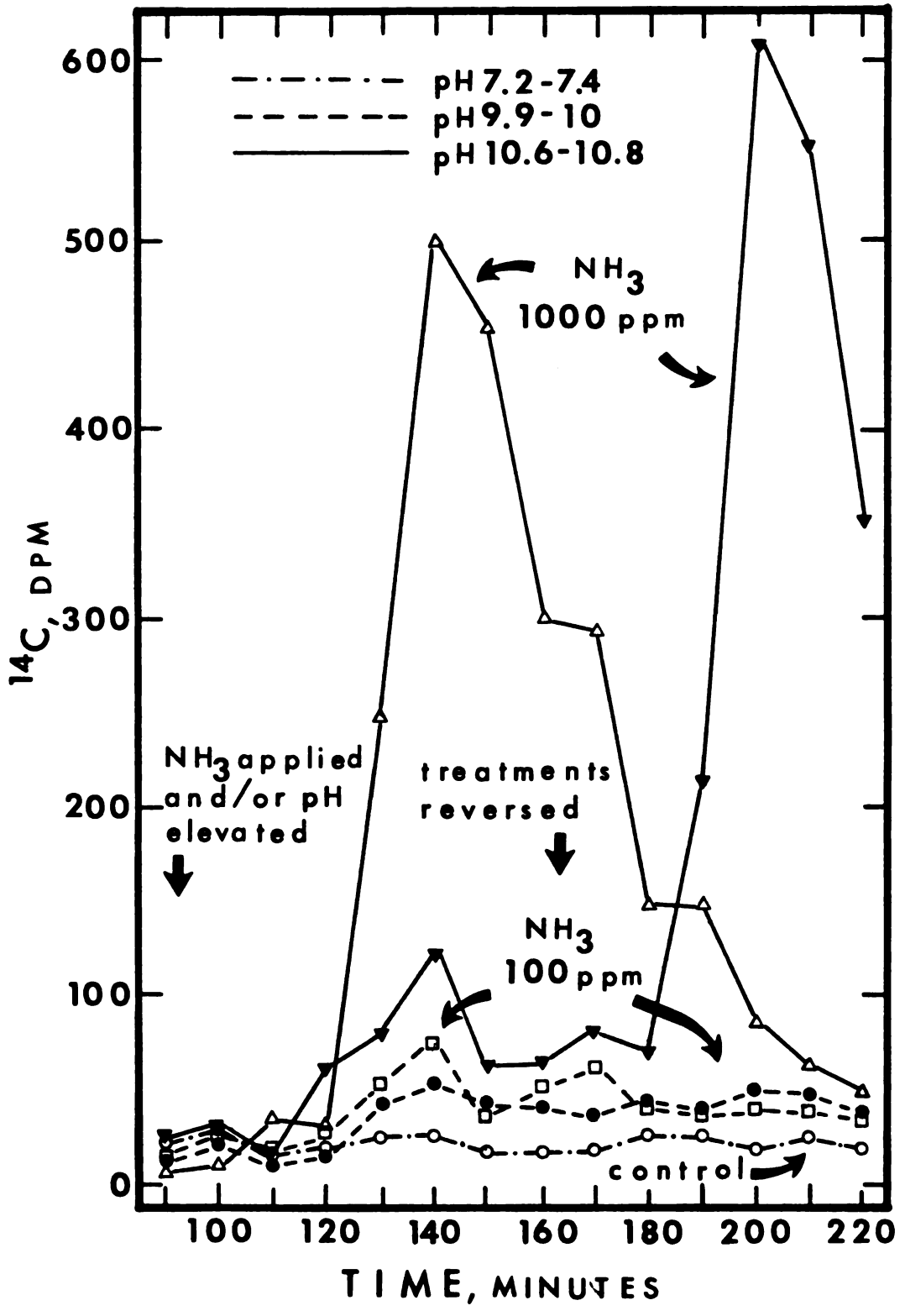
Figure 19. Effect of ammonia from NH_4OH, at concentrations of 100, 500, and 1000 mg/L (ppm), and the effect of alkaline pH's on viability of sclerotia of M. phaseolina in a static system for 5 days. CFU, colony-forming units.


Macrophomina phaseolina, CFU
150
100
50
0
0
2
4
6
8
120
HOURS
Ammonia
No Ammonia
pH 11.2
control
pH 5.2
500 ppm
(pH 10.7)
100 ppm
(pH 10.0)
1000ppm
(pH 10.8)
pH 11.9

6.3.5 ^{14}C-labeled exudation from conidia exposed to high pH and ammonia levels on leached sand.

^{14}C-labeled exudation from C. victoriae conidia incubated on sand leached with solutions at pH 10 and 10.7 without ammonia was increased only slightly over the control (pH 7.2-7.4) values (Fig. 20). At pH 10, the amount of exudate was about twice that of the control, but the conidia exposed to the same pH with 100 mg NH_3/L exuded almost three times as much exudate as the control during the same period. The pH 10.8 solution caused a greater loss of ^{14}C-labeled exudate, about five times more than the control. However, the addition of ammonia (1000 mg/L) at about the same pH caused a rapid increase of exudation, resulting in a flush of exudation about 19 times that of the control. When the solutions were switched, little change was observed at pH 10 with or without ammonia; the pH 10.8 solution did not stimulate exudation, whereas 1000 mg NH_3/L at pH 10.7, following the pH 10.8 buffer alone, again greatly stimulated exudation.

Figure 20. Total activity exuded from ^{14}C-labeled conidia of C. victoriae on the leaching system treated with alkaline solutions of pH 7.2-7.4, 9.9-10, and 10.6-10.8 and with the same alkaline solutions but with 0, 100, and 1000 mg/L (ppm) of ammonia from NH_4OH, respectively. The ammonia-supplemented solutions were switched with the corresponding alkaline solutions, after 160 min.


pH 7.2-7.4
pH 9.9-10
pH 10.6-10.8
NH3 1000 ppm
NH3 applied and/or pH elevated
treatments reversed
NH3 100 ppm
control
14C, DPM
TIME, MINUTES
600
500
400
300
200
100
0
100
120
140
160
180
200
220

6.3.6 ^{14}C-labeled exudation from conidia not under nutrient stress and exposed to high pH and ammonia levels in a static system.

C. sativus conidia were exposed to increasing ammonia concentrations at pH 9.2, 10.1 or 11.1. ^{14}C-labeled exudation over 4 h was greater as pH, without ammonia, increased (Fig. 21). Within each pH series, exudation was reduced with increasing concentrations of ammonia until about 50-100 mg NH_3/L, whereas at 100-1000 mg NH_3/L, this trend was reversed and exudation was increased two to three times over that without ammonia. Only at pH 9.2 was the increased exudation at 1000 mg/L less than that without ammonia.

The viability of conidia was inversely related to the increased exudation occurring at the higher ammonia concentrations (Fig. 22). High pH per se did not reduce the viability of the conidia. Viability dropped to zero at pH 11.1 with 250 mg NH_3/L, at pH 10.1 with 500 mg NH_3/L, and at pH 9.2 with 750 mg NH_3/L.

Figure 21. Percentage of total activity exuded from ^{14}C labeled conidia of C. sativus in a static system treated with alkaline solutions of pH 6.7-8.6, 9.2, 10.1, and 11.1 and ammonia concentrations of 0-1000 mg/L (ppm), from NH_4Cl. The control pH series (6.7-8.6) contained as much NH_4Cl as the pH 9.2 series.

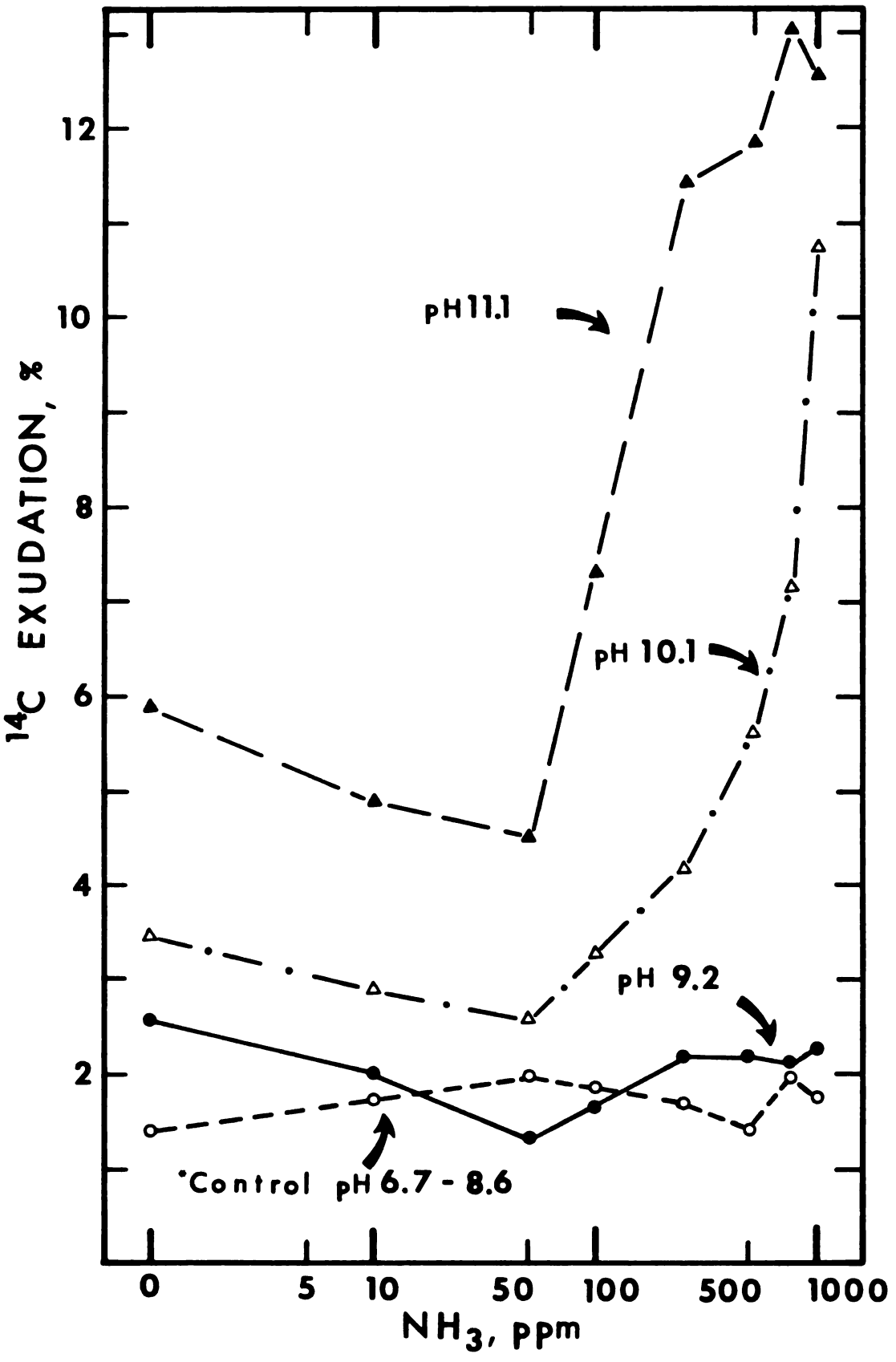
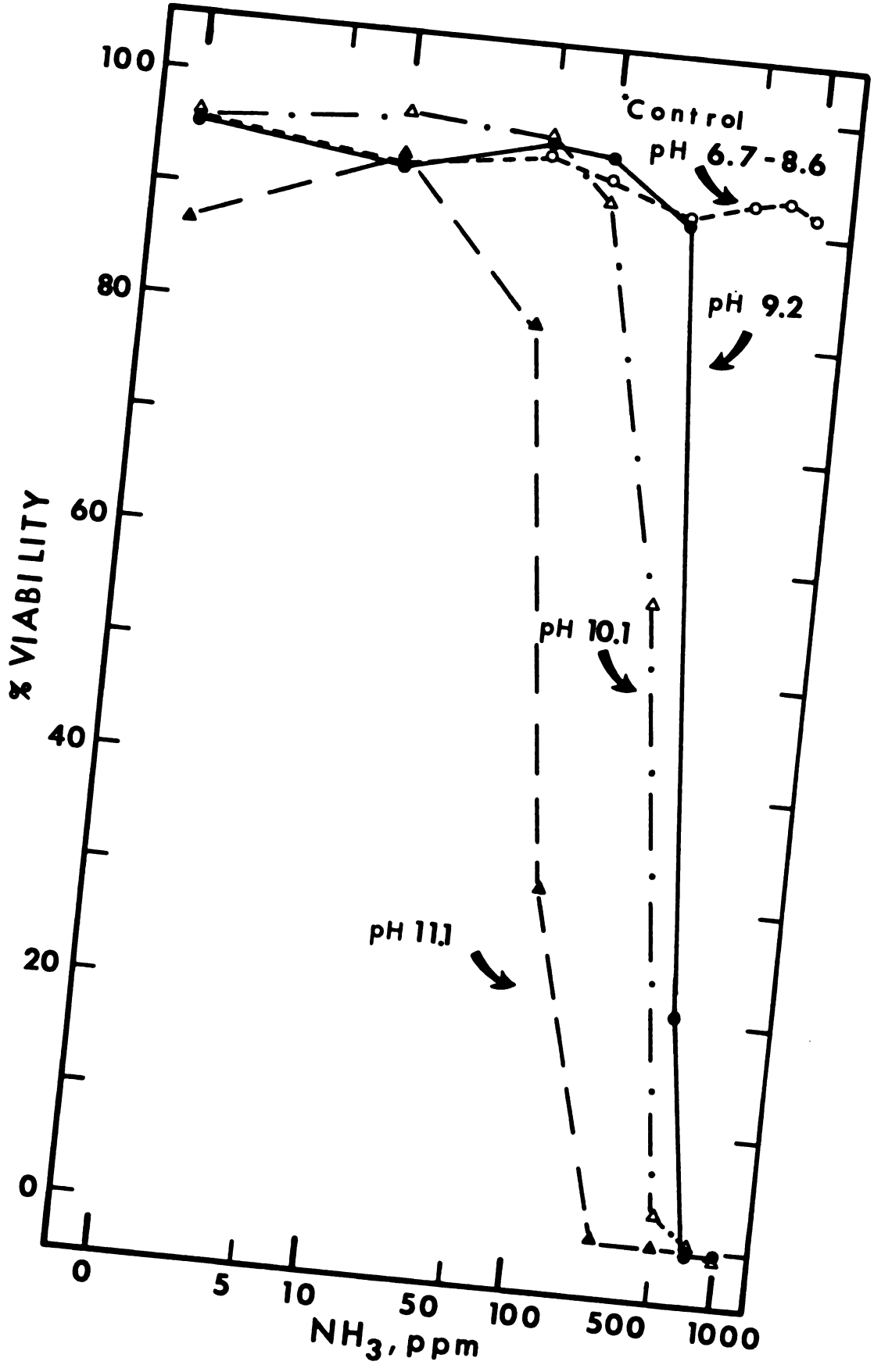

12
10
8
6
4
2
^{14}C EXUDATION, %
pH 11.1
pH 10.1
pH 9.2
*Control pH 6.7 - 8.6
0
5
10
50
100
500
1000
NH_3, ppm

Figure 22. Viability of ^{14}C labeled conidia of C. sativus in a static system treated with alkaline solutions of pH 6.7-8.6, 9.2, 10.1, and 11.1 and increasing ammonia concentrations of 0-1000 mg/L (ppm) from NH_4Cl. The control pH series (6.7-8.6) contained as much NH_4Cl as the pH 9.2 series.


100
80
60
40
20
0
% VIABILITY
Control
pH 6.7 - 8.6
pH 9.2
pH 10.1
pH 11.1
0
5
10
50
100
500
1000
NH3, ppm

6.4 DISCUSSION

The rapid increase of pH when soil was supplemented with finely divided urea particles indicates that the urea conversion to ammonia was a very rapid process. After 24 h respiration of the soil microflora was repressed more at 5% than at 1% urea. This repression, which was observed as early as 4 h, was probably due to low levels of ammonia since urea itself has low toxicity to many fungal propagules (D. Chun, unpublished results), and the soil pH was too low to be inhibitory by itself or to allow a large proportion of ammonia to be in the nonionized form (Eno et al., 1955; Henis and Chet, 1967; Rush, 1981; Smith, 1964).

In the urea treatments, a greater proportion of ^{14}C-labeled exudate from propagules was left in the soil as unrespired ^{14}C than was the case in control soil, probably reflecting repressed soil respiration owing to ammonia. A greater proportion of ^{14}C-labeled exudate from sclerotia of M. phaseolina remained unrespired in soil than was the case with conidia of C. victoriae. This was probably due to greater exudation by C. victoriae and or to differences in the composition of exudates of the two fungi. The sclerotia also exuded much less than the conidia, as was shown previously (Filonow and Lockwood, 1983a; 1983b), and this may relate to the greater longevity in soil of M. phaseolina (Bhattacharya and Samaddar, 1976). Since ammonia in low concentrations inhibited respiration in mycelium of Phymatotrichum omnivorum

(Rush and Lyda, 1982), there is no reason to believe that the increased respiration was from the labeled conidia.

The greater exudation by C. victoriae conidia at 1% than at 5% urea was probably because a lethal concentration of ammonia was achieved more rapidly at 5% urea. Since pH was highest at the higher concentration of urea, a direct effect of increasing pH on exudation is precluded. If exudation involves metabolic activity, higher concentrations of ammonia may quickly inactivate the processes involved, whereas at lower concentrations, damage may be less or occur more slowly. In contrast, exudation by M. phaseolina was increased more in soil with 5% than with 1% urea.

Exudation from conidia of Cochliobolus victoriae was also increased by ammonia on leached sand. Conidia exuded more in a solution containing 1,000 mg than from 10,000 mg NH_3/L, which follows the trend observed in soil, and probably occurred for the same reason. The rapid increase in exudation followed by a rapid decline seem to be characteristic of killed propagules. The rate of exudation from conidia made nutrient-dependent was much lower than that from nutrient-independent conidia. However, in the presence of sublethal ammonia concentrations in short-term experiments, exudation from nutrient-dependent conidia increased dramatically and remained elevated over the controls. Leakage from sclerotia of M. phaseolina on leached sand was greatest at high concentrations, as in soil, and in short-term experiments, viability was reduced only at very high concentrations. Concentrations that were sub-lethal in short-term experiments, however, reduced viability when the duration of exposure was longer, as was the

case with C. sativus.

Elevated pH alone stimulated exudation by C. sativus in the static system and by C. victoriae in the leaching system but did not account for the loss of viability. In the static system, at low concentrations (<100 mg/L), ammonia appears to have a sparing effect on exudation. This was also observed by H. Löffler and B. Schippers with Fusarium solani f.sp. phaseoli, C. victoriae, and Botrytis cinerea (personal communication). This sparing effect may increase survival of the propagules in alkaline soils. However, as ammonia concentration increased, a point was reached where exudation was stimulated. The increased exudation was associated with decreased viability, both of which were highly influenced by the pH of the system.

Little is known about the biological effects of volatile inhibitors in soil. Where ammonia was applied to soil at relatively high levels (Eno et al., 1955; Rush, 1981), the populations of soil organisms declined. Ammonia-ammonium enters and leaves the cells freely and may accumulate internally depending on the external concentration (Macmillan, 1956) and at high concentrations may damage fungal membranes (Rush and Lyda, 1982). Ammonia at low concentrations has been described by many workers as having a fungistatic role in soil (Ko et al., 1974; Pavlica et al., 1978; Schippers and Palm, 1973). While a sparing effect on exudation has been observed under a low nutrient stress environment, soil acts as a strong nutrient sink on fungi (Filonow and Lockwood, 1983a; 1983b) occurring under nutrient stress (Filonow et al., 1983; Filonow and Lockwood, 1983b), the possible acceleration of these processes by ammonia is intriguing, especially

since low concentrations of ammonia resulted in the slow decline of populations of Pythium ultimum, Thielaviopsis basicola, and M. phaseolina in soil (Chun and Lockwood, 1982).

Chapter 7

GENERAL DISCUSSION

Plant diseases are influenced in many different ways by nitrogen fertilizers (Huber and Watson, 1974). While in some cases the effect is directly on the plants affected, most work on soil-borne plant diseases has centered on the influence of the amendment directly or indirectly on the pathogens involved. In studies in which organic amendments with high C:N ratios have been used, increased competition for nitrogen can explain disease reduction (Garrett, 1970; Lindsey, 1965; Maurer and Baker, 1965; Snyder et al., 1959). Where nitrogen was limiting, Fusarium solani f. sp. phaseoli was less active as a pathogen (Snyder et al., 1959), but when more nitrogen was made available, the pathogen became active and caused severe disease (Maurer and Baker, 1965). Microbial activity may become increased following introduction of organic amendments to soil. This may lead to increased soil fungistasis (Adams and Papavizas, 1969) and reduced disease (Papavizas and Adams, 1969; Papavizas et al., 1970). Where some nutrients are limiting, increased demand may further limit availability. But not all soil-borne pathogens are influenced the same way. When soil infested with Thielaviopsis basicola was amended with crop residues, the population density of the pathogen decreased due to stimulation of germination of endoconidia and chlamydospores with subsequent germling

lysis before secondary endoconidia or chlamydospores could be formed (Lewis and Papavizas, 1975; Sneh et al., 1976). While work with Fusarium populations suggested that organic amendments with high C:N ratios were more effective than organic amendments with low C:N ratios in reducing disease severity or population densities of the pathogen (Lewis and Papavizas, 1975); Maurer and Baker, 1965; Snyder et al., 1959), other work began to suggest that low C:N ratio amendments were also effective (Chun and Lockwood, 1982; Filonow and Chun, 1981; Gilpatrick, 1969a, 1969b; Schippers and Palm, 1973; Tsao and Zentmeyer, 1979; Zakaria et al., 1980). Zakaria et al. (1980) demonstrated that volatile degradation products of various oilseed meal amendments decreased Fusarium populations in soil and that the principle volatile involved was ammonia. Propagule reduction by ammonia-yielding amendments was not due to competition or germination-lysis but by direct killing by ammonia (Rush and Lyda, 1982a; 1982b; Schippers and Palm, 1973; Tsao and Zentmyer, 1979).

While P. ultimum is generally considered to be a weak soil saprophyte, it is quite capable of colonizing a readily available food source and increasing its population, which was the case in the field trials and laboratory trials using open containers. Soybean meal consistently reduced P. ultimum populations in laboratory experiments in closed containers (Filonow and Chun, 1981); even in open containers, where P. ultimum population showed an initial increase, the population dropped shortly after to very low or undetectable levels. In the field, however, colonization occurred without subsequent decrease until 26 days post-application and the reduction was not as large as in the

laboratory. Lethal effects of ammonia may have occurred, but the increased population size may have masked any reduction. The initial increase in population density was easily circumvented by replacing soybean meal with urea.

In soil, urea undergoes hydrolysis to generate ammonia which may undergo further conversion to nitrate. Nitrate and urea itself were slightly inhibitory to the fungal propagules tested at high concentrations, but could not account for the population reductions observed in laboratory or field trials. Urea was effective in reducing soil populations of T. basicola, P. ultimum, and M. phaseolina to very low levels in laboratory and field experiments. However, its widespread use for this purpose may be prohibitively expensive. At the current price of $ .248/kg of urea, the approximate cost would be $562-703 per acre ($1406-1758 per hectare) to treat a field at the 0.25% rate to 6-7 in. depth which is equivalent to about 2.5-3.1 tons per acre (5.7-7.1 metric tons per hectare). Further research might focus on urea application in localized areas of pathogen infestation or where low concentrations can be applied that might bring about a gradual population decline. Results indicate that the lowest effective urea concentration is between 0.1% and 0.25%, the choice being dependent on the pathogen, the soil temperature, and how quickly the population is to be reduced. Where conditions warrent, urea at 0.1% or a higher concentration applied to a localized area would substantially reduce the treatment cost. An added benefit would be that nitrogen fertilization is being accomplished at the same time. Although inhibition of seed germination was not observed in soil a month after application of 0.1

and 1% urea, plant growth may be affected by the alterations of soil pH (and possibly of soil structure), high concentrations of soil nitrogen, and changes in the microbial flora. Further work should be continued along these lines.

Soil pH increased rapidly when soil was supplemented with urea indicating that conversion to ammonia was a rapid process. Respiration of the soil microflora was repressed as early as 4 h after urea was added to soil. Since 4 h was insufficient time for the soil pH to increase to inhibitory levels or to allow a large proportion of ammonia to be in the un-ionized form (Eno et al., 1955; Henis and Chet, 1967; Rush, 1981; Smith, 1964), and urea by itself has low toxicity, repression of respiration was attributed to low concentrations of ammonia.

Exudation from sclerotia of M. phaseolina and from conidia of C. victoriae was increased in urea-amended soils. The greater rate of exudation with conidia than with sclerotia has been shown previously (Filonow and Lockwood, 1983a; 1983b), and this may relate to the greater longevity in soil of M. phaseolina (Bhattacharya and Samaddar, 1976). Exudation by fungal propagules was also increased by ammonia on leached sand. The rapid increase in exudation followed by a rapid decline at high ammonia concentrations (10^3-10^4 mg/L), seems to be characteristic of killed propagules. Short-term exposure to sublethal doses of ammonia also stimulated exudation by nutrient-independent and nutrient-dependent conidia of C. victoriae. Leakage from sclerotia of M. phaseolina on leached sand increased with increasing ammonia concentrations, as in soil. In short-term experiments, viability was reduced

only at very high concentrations, but extended exposure to sublethal doses of ammonia resulted in reduced viability.

Exudation by the fungal propagules may not be entirely passive. On leached sand, the conidia exuded more in the presence of 1,000 mg NH_3/L than from 10,000 mg, probably due to more rapid inactivation of metabolic activities by the higher concentration of ammonia. Likewise, the greater exudation at a lower than at a higher concentration of urea by the conidia in soil may have occurred because a toxic concentration of ammonia was reached more rapidly with the higher urea application.

Elevated pH alone stimulated exudation by C. sativus in the static system and by C. victoriae in the leaching system, but did not account for the loss of viability. In the static system, at low concentrations (<100 mg/L), ammonia appeared to have a sparing effect on exudation. This was also observed by H. Löffler and B. Schippers with Fusarium solani f. sp. phaseoli, C. victoriae, and Botrytis cinerea (personal communication). Possibly this sparing effect may increase survival of the propagules in alkaline soils. However, as ammonia concentration increased, a point was reached where exudation was stimulated. The increased exudation was associated with decreased viability, both of which were highly influenced by the pH of the system. The effect of ammonia on the ultrastructure and physiology of the fungal propagules should be explored with differential biochemical staining of subcellular components and radioactive labeling to better determine the role of ammonia and pH. Possibly with more sensitive ammonia electrodes, the route of ammonia under different pH's can be followed through the cell.

Little is known about the biological effects of volatile inhibitors in soil. Where ammonia was applied to soil at relatively high concentrations (Eno et al., 1955; Rush, 1981), the populations of soil organisms declined. Ammonia-ammonium enters and leaves the cells freely and may accumulate internally depending on the external concentration (Macmillan, 1956) and at high concentrations may damage fungal membranes (Rush and Lyda, 1982). Ammonia at low concentrations has been described by many workers as having a fungistatic role in soil (Ko et al., 1974; Pavlica et al., 1978; Schippers and Palm, 1973). While a sparing effect on exudation has been observed under a low nutrient stress environment, soil acts as a strong nutrient sink on fungi (Filonow and Lockwood, 1983a; 1983b), and under such conditions exudation in vitro was increased over an extended period. In view of the decreased germinability and virulence of C. victoriae occurring under nutrient stress (Filonow et al., 1983; Filonow and Lockwood, 1983b), the possible acceleration of these processes by ammonia is intriguing, especially since low concentrations of ammonia resulted in the slow decline of populations of P. ultimum, T. basicola, and M. phaseolina in soil (Chun and Lockwood, 1982). Where soils are suppressive to diseases caused by soil-infecting pathogens, would urea or ammonia application destroy this suppressiveness, improve or create suppressive soils to disease, or have no effect at all? The same questions could be asked of conducive soils. Therefore, additional study on the effects of ammonia on soil ecology, particularly in regard to long term influences on the soil microflora with single or repeated applications, is still needed.

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