Reductions of *Pythium ultimum, Thielaviopsis basicola*, and *Macrophomina phaseolina* Populations in Soil Associated with Ammonia Generated from Urea

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ABSTRACT

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Sandy loam soil infested with *Pythium ultimum, Thielaviopsis basicola*, and *Macrophomina phaseolina* was amended with urea at concentrations of 0.1, 0.25, 0.5, and 1.0 (w/w) in field microplots. Ammonia concentrations increased with the amount of urea applied. Population densities of all three pathogens were significantly and often markedly decreased by urea concentrations of 1.0, 0.5, and 0.25%; 0.1% urea appeared to be effective in reducing soil populations after 31 days at high soil temperatures but was ineffective even after 34 days at low soil temperatures.

Additional key words: soil amendments, soilborne pathogens

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 (9). Certain nitrogenous materials showing fungicidal activity in vitro (2,7,8,16,22) have been used in attempts to control soilborne pathogens with both positive (2,20) and negative (18,21) results.

Zakaria and Lockwood (21) found that oilseed meals were effective as soil amendments to reduce *Fusarium* populations in the laboratory. Subsequent work (22) demonstrated that oilseed meals were more effective in closed than in open containers and that *Fusarium* populations were reduced in soil in planchets incubated on the surface of oilseed meal-amended soils in closed containers. These results strongly implicated one or more volatile inhibitors from these amendments. 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. The relative effectiveness of the amendments was directly correlated with the amount of ammonia produced upon decomposition of the amendment (22). Oilseed meals, however, failed to reduce *Fusarium* populations in covered or uncovered plots in the field (21).

Soybean meal amendment also reduced populations of *Pythium ultimum* Trow. under both covered and uncovered conditions in the laboratory (2). Under field conditions, however, the pathogen colonized the soybean meal; its population increased initially, then decreased but frequently to levels no less than that of the original population. To circumvent the problem of colonization of the amendment by the pathogen, urea was tested in the laboratory as an alternate source of ammonia and was found to reduce populations of *P. ultimum* without an initial increase.

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

MATERIALS AND METHODS

Fungi and preparation of inocula. *P. ultimum* was isolated from soybean seedling pieces (17). *M. phaseolina* isolate 4 was obtained from T. D. Wyllie (Department of Plant Pathology, University of Missouri, Columbia). *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) made by adding 5 g of Difco yeast extract per liter of PDA. A liter of carrot agar was prepared by autoclaving 30 g of sliced carrots in 250 ml of distilled H_2O, then decanting the supernatant solution into 20 g of agar in 750 ml of distilled H_2O.

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, IL). *M. phaseolina* and *T. basicola* were cultured in 500 ml of PD broth and PDY broth, respectively, in aluminum pans (50 × 29 × 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 wk.

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 at 6,400 rpm for 30–60 sec and at 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. Propagules of all three pathogens were mixed into smaller amounts of soil for later incorporation. These stock soils were kept moist at 24–27 C for at least a week to permit lysis of mycelial fragments, then air-dried and thoroughly mixed together.

Assays for fungal populations. Populations of *P. ultimum* were determined by the method of Stanghellini and Hancock (19), modified 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 (2). Soil dilutions were dispensed into wells 2.5 mm in diameter × 2 mm deep made in 2% water agar plates (50–52 wells per plate) at about 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 and soil pH and ammonia concentrations were determined in the microplots. Soil samples were kept on ice until assayed, usually within 1 hr of 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

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determine soil moisture content (sample dried overnight at 90 C). To assay populations of *P. ultimum* and *T. basicola*, soil was suspended in 0.2% water agar and shaken for 1 hr before dilutions were made in 0.2% water agar. A selective medium for *T. basicola* (TBM-C medium), made with carrot decoction, was poured into plates containing soil dilutions to estimate populations of *T. basicola* (11). *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 (2). Soil samples were dispersed in 60% (w/w) sucrose solution and centrifuged for 10 min at 3,100 *g*. 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 surface-sterilized in 0.5% NaOCl for 5 min, rinsed, and suspended and diluted in 0.2% water agar. Selective medium (12) was poured into plates containing sclerotial dilutions, and colonies were counted after 7–10 days of incubation at 30 C.

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. Microplots consisted of cylindrical clay drainage tiles 20.3 cm in diameter × 30.5 cm deep. Each tile was sunk about 28 cm into the soil and 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 infested with propagules of the three pathogens. The infested soil was Marlette or Boyer sandy loam (1981 trials) or Boyer sandy loam (1982 trial). 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 six 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 analyzed 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 were injected into a Varian Aerograph 1400 series gas chromatograph (Varian Instruments, Palo Alto, CA) equipped with a glass column (1.83 m × 0.64 mm × 2 mm i.d.) packed with Chromosorb 103 (Johns-Manville, Denver, CO) in trial 2. In trial 3, concentrations of ammonia in the boric acid solution were determined spectrophotometrically using Nessler's reaction (6). Ammonia concentrations are expressed on a soil dry weight basis.

Urea granules (Mallinckrodt, Inc., Paris, KY) 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 as a control. Treatments were randomized; 12 replicates were used for the 1981 trials and eight 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.

RESULTS

In the first trial (Fig. 1A), the population of *P. ultimum* remained relatively stable in control microplots, ranging from 128 to 226 colony-forming units per gram of soil (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 12 days, *P. ultimum* was not detected.

The population of *T. basicola* remained stable in the controls at about 20,000

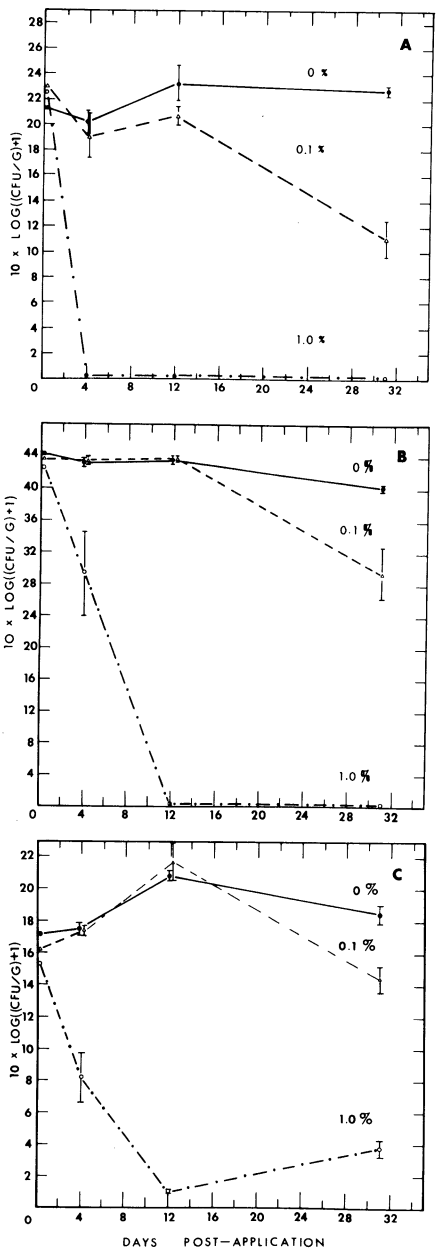

Fig. 1. Effects of 0, 0.1, and 1.0% urea amendment on soil populations of **(A)** *Pythium ultimum*, **(B)** *Thielaviopsis basicola*, and **(C)** *Macrophomina phaseolina* after 4, 12, and 31 days in microplots in the field. Trial 1 was conducted from 17 July to 17 August 1981. Vertical bars represent one standard error.

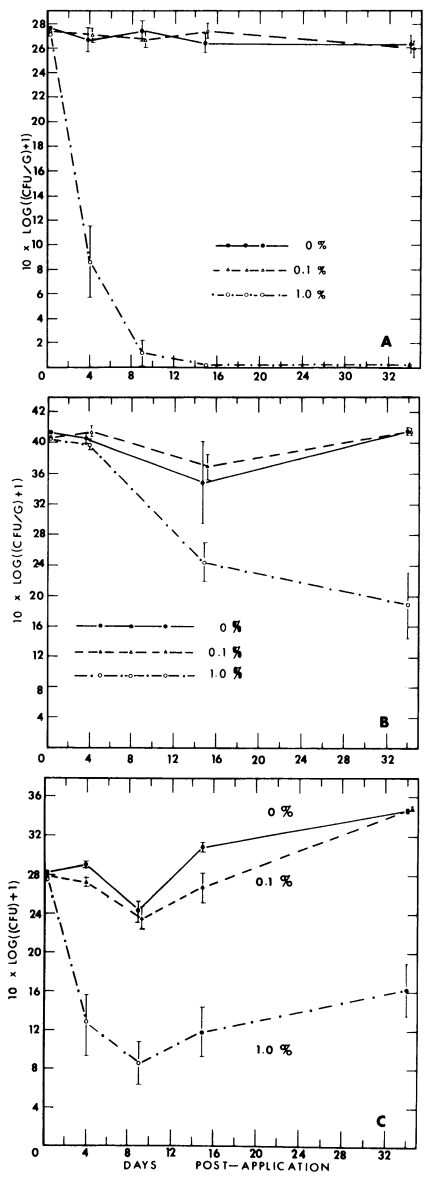

Fig. 2. Effects of 0, 0.1, and 1.0% urea amendment on soil populations of **(A)** *Pythium ultimum*, **(B)** *Thielaviopsis basicola*, and **(C)** *Macrophomina phaseolina* after 4, 9, 15, and 34 days in microplots in the field. Trial 2 was conducted from 24 September to 28 October 1981. Vertical bars represent one standard error.

cfu/g (Fig. 1B). 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. 1C) by 31 days. With 1.0% urea, the population of *M. phaseolina* dropped to about 11 cfu/g after 4 days and to 1 cfu/g after 12 and 31 days.

During the second trial, pathogen populations were not reduced as rapidly or as drastically by 1.0% urea as in the first experiment; 0.1% urea did not significantly decrease the fungal populations (Fig. 2). Trial 2 was conducted from late September through October, when soil temperatures were often 8–10 C lower than those in trial 1 (Fig. 3). 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. 4A). 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 1,900 mg/kg after 4 days, then decreased by the end of the experiment to about 500–600 mg/kg. Soil treated with 0.1% urea did not exceed 80 mg/kg (Fig. 4B).

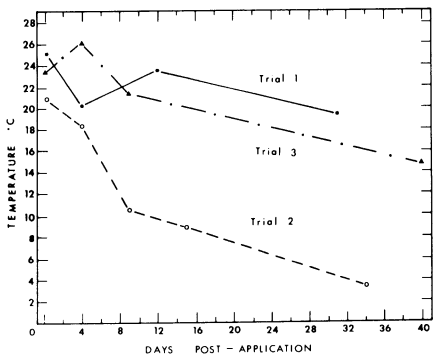

Fig. 3. Soil temperature 7–8 cm below the soil surface taken during trials 1, 2, and 3 conducted from 17 July to 17 August 1981, 24 September to 28 October 1981, and 21 July to 30 August 1982, respectively.

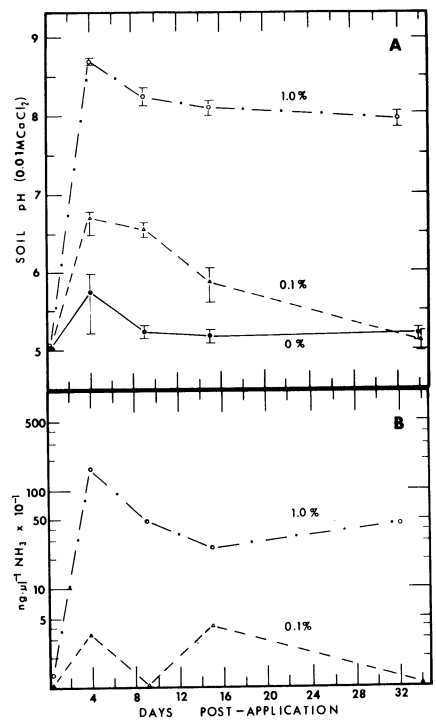

Fig. 4. Effects of 0, 0.1, and 1.0% urea amendment of soil on **(A)** soil pH (vertical bars represent one standard error), and **(B)** soil NH_3/NH_4^+ concentration (background NH_3/NH_4^+ subtracted). Trial 2 was conducted from 24 September to 28 October 1981.

During trial 3, populations of *P. ultimum* in untreated soil remained stable at about 190 cfu/g during the experiment (Fig. 5A). 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.

The population of *T. basicola* in untreated soil remained relatively constant at about 10^5 cfu/g (Fig. 5B). In soil treated with 0.25% urea, the population dropped to less than 0.17 of its initial size after 9 days, and by 40 days, it 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.

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. 5C). With 0.5% urea, the population was reduced to 0.02 of the initial population by 4 days, and by 40 days, it was barely detectable. With 1.0% urea, the population dropped to zero within 9 days.

Trial 3 was run from late July until the end of August. Soil temperatures ranged from a high of 26 C to a low of 15 C at the end of the experiment (Fig. 3). Ammonia levels in control soils remained constant at about 8–10 mg/kg (Fig. 6). 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 and 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.

DISCUSSION

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. *P. 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. 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 (1) 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 (2) may also have been due to low soil temperature, because this experiment was also done in the fall. These results are consistent with the observation of Papavizas et al (13) that bean root rot caused by *T. 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 (2). 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 (20) and of *Rhizoctonia solani* by plant residues of low C:N ratio (10). Anhydrous ammonia is toxic to a number of fungi (14,15,18).

The mode of action of ammonia in reducing pathogen populations in soil is uncertain. Reductions in populations of *P. cinnamomi* and *P. 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 (20). In other work in our laboratory, ammonia stimulated propagule exudation in vitro and this was associated with death of the propagules (2,3). Moreover, exposure to concentrations of ammonia that were nonlethal during shorter exposures

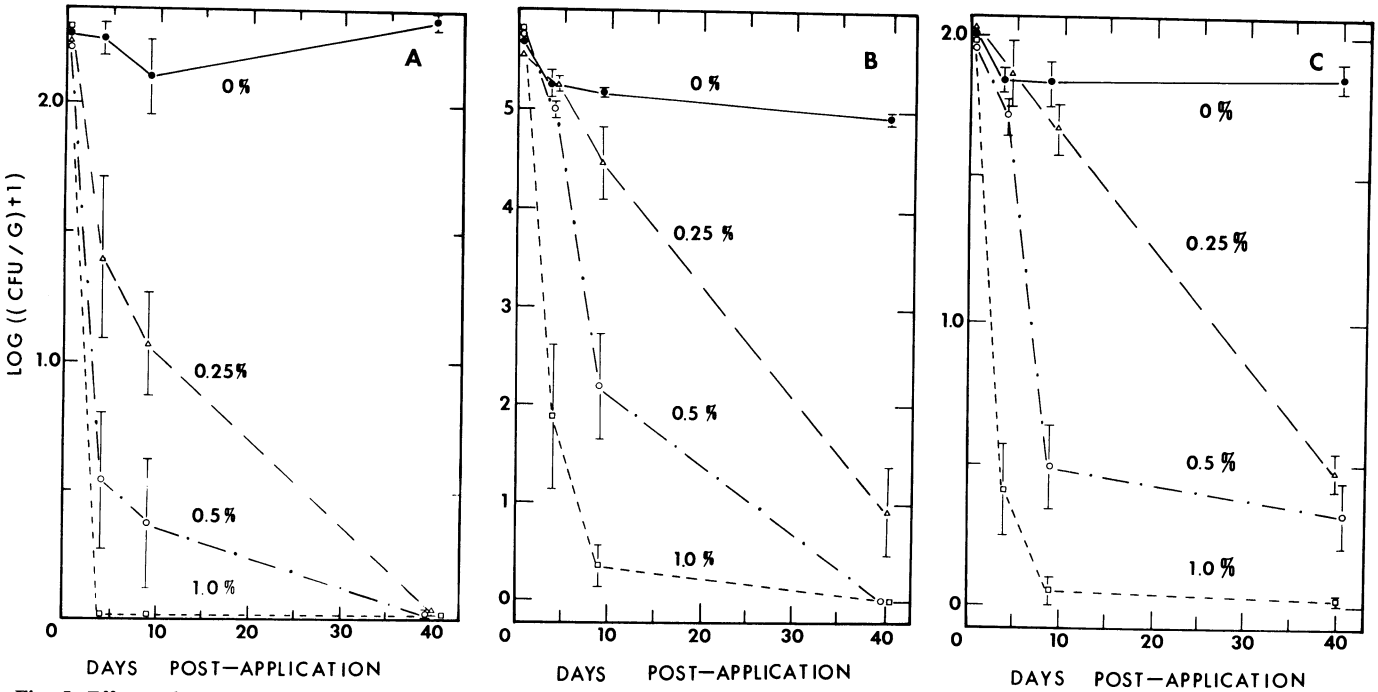

Fig. 5. Effects of 0, 0.25, 0.5, and 1.0% urea amendment on soil populations of **(A)** *Pythium ultimum*, **(B)** *Thielaviopsis basicola*, and **(C)** *Macrophomina phaseolina* after 4, 9, and 40 days in microplots in the field. Trial 3 was conducted from 21 July to 30 August 1982. Vertical bars represent one standard error.

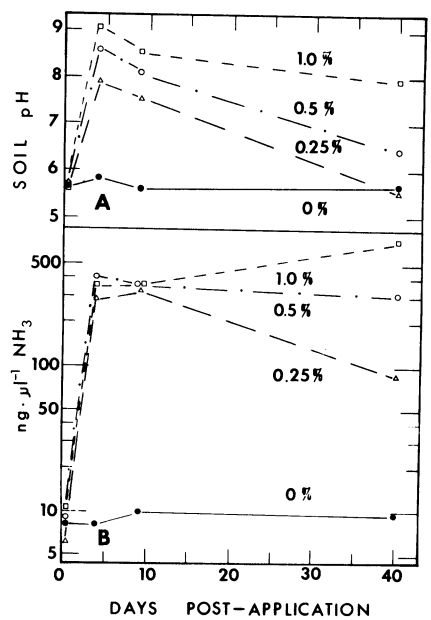

Fig. 6. Effects of 0, 0.25, 0.5, and 1.0% urea amendment of soil on **(A)** soil pH, and **(B)** soil NH_3/NH_4^+ concentration. Trial 3 was conducted from 21 July to 30 August 1982.

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 or for its lethal effects (3). 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 (5). Ammonia may have been involved in the long-term decline in populations of *M. phaseolina* sclerotia occurring in nitrogen-amended soil in sealed containers (4).

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 $0.25 per kilogram of urea, the approximate cost would be $562–703 per acre ($1,406–1,758 per hectare) to treat a field at the 0.25% rate to a depth of 15.2–17.8 cm. Further research might focus on application of urea in localized areas of pathogen infestation or where low concentrations can be applied that might bring about a gradual population decline and also 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.

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