Effects of Anhydrous Ammonia on Mycelium and Sclerotia of *Phymatotrichum omnivorum*

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ABSTRACT

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Anhydrous ammonia (NH_3) was toxic to mycelium and sclerotia of *Phymatotrichum omnivorum*. Mycelium was much more sensitive than sclerotia to NH_3, and a 1-min exposure to 4 μg NH_3/ml was lethal. Mycelium in naturally infected root pieces was killed within 24 hr by exposure to 56 μg/ml NH_3. Shorter exposures resulted in increased survival of mycelium in roots. NH_3 concentrations below 21 μg/ml were not toxic to sclerotia in vitro even after a 48 hr of exposure. Toxicity at 28 μg/ml was proportional to exposure period, giving 3, 23, 34, and 59% of kill of sclerotia after 1, 12, 24, and 48 hr. Exposure of sclerotia to NH_3 concentrations of 42, 56, or 84 μg/ml for 12 hr resulted in 100% kill in vitro. Higher concentrations of NH_3 were required to achieve toxicity to sclerotia in situ; 138 and 276 μg/g resulted in 35 and 79% kill, respectively. Electrolyte leakage from mycelium increased in proportion to NH_3 concentration after the first 15 min. Mycelial respiration was highly sensitive to NH_3, and was inhibited 55% by 4 μg/ml and 82% by 8 μg/ml.

The fungicidal action of ammonia on various genera of fungi has been reported by numerous authors (4–7,11,14,27,30). Many of these reports were concerned with the toxicity of liberated ammonia gas from soil or organic amendments (4,5,8,26,29), to the toxicity of anhydrous ammonia, NH_3 (2,3,6,22,23), or ammonia liberated from nitrogenous compounds such as NH_4OH, NH_4Cl, or $(NH_4)_2SO_4$ (12,13,18,24,25). The conclusion common to the majority of these papers was that ammonia gas is toxic in low concentrations (1–100 μg/g air) and to a certain extent, reduces disease severity. Neal et al (16) first investigated the toxicity of ammonia, ammonium salts, and NH_4OH to *Phymatotrichum omnivorum* in 1933. They reported a 100% kill of sclerotia of *P. omnivorum* after a 20-sec exposure to ammonia gas generated from 200 ml of 28% NH_4OH. Neal and Collins (15) reported that low

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concentrations of ammonia and NH_4OH were toxic to the fungus in vitro, but extremely high concentrations were required for control in the soil. Because of this, plus findings that NH_4^+ salts were not toxic to the fungus (26), investigation of compounds as control agents for Phymatotrichum root rot was discontinued.

The use of anhydrous ammonia or ammonia-generating compounds as controls for soilborne pathogens has usually met with limited success because of inconvenience, expense, or lack of movement of ammonia in the soil. Unlike most soilborne pathogens, *P. omnivorum* survives in localized areas in the field. The fact that anhydrous ammonia is toxic to many fungi and also is very inexpensive suggested its use in spot treatments for control of Phymatotrichum root rot.

The purpose of the research reported in this paper was to determine the levels of anhydrous ammonia toxic to *P. omnivorum* and to describe some of its effects on the fungus.

MATERIALS AND METHODS

Fungal cultures. Sclerotia were produced in the laboratory as previously described (19). Houston black clay (250 g), sorghum seed (50 g), and distilled H_2O (50 ml) were placed in Erlenmeyer flasks (500 ml) and autoclaved for 1 hr at 121 C and 103.4 kPa (15 psi). Mycelial plugs of *P. omnivorum* from potato-dextrose agar (PDA) plates were introduced into these flasks. By using this method, mature sclerotia were produced in 3–4 wk.

Mycelial mats of *P. omnivorum* were produced in a liquid medium consisting of commercially canned carrot juice (200 ml), dextrose (20 g), and streptomycin sulfate (200 μg/ml). Mycelial mats weighing approximately 400 mg (dry weight) per flask were obtained within 10 days by introducing 25 ml of medium per 125-ml Erlenmeyer flask with a mycelial plug (1 cm) from PDA culture. Cultures were incubated at 28 C in stationary culture. New cultures were started every 2 wk to maintain a continuous supply of mycelium of approximately the same age.

TABLE 1. Germination of *Phymatotrichum omnivorum* sclerotia in vitro as a function of NH_3 concentration and exposure time

NH_3 (μg/ml)	Germination (%) after exposure for:			
	1 hr	12 hr	24 hr	48 hr
0	100[a]	100	100	100
14	100	100	100	100
21	100	100	97	88
28	97	77	66	41
42	52	0	0	0
56	33	0	0	0
84	0	0	0	0

[a] Percent germination of 40 sclerotia 7 days after being plated on.

Toxicity of NH_3 to *P. omnivorum* sclerotia in vitro. Sclerotia were washed with tap water from soil-culture flasks, blotted, and dried for 1 hr at 25 C; approximately 1 g was placed in glass tubes and sealed with rubber caps. Varying amounts of anhydrous NH_3 (Scientific Gas Products, Pasadena, TX 77502) were injected into the tubes with a Gilson gas syringe and allowed to incubate with the sclerotia for different periods of time. The NH_3 concentrations used in this experiment were 14, 21, 28, 56, and 84 μg NH_3/ml of air, and the exposure periods were 1, 12, 24, and 48 hr. Five replications of each NH_3 concentration were tested at each exposure period. Immediately after exposure, sclerotia were washed in distilled H_2O, surface-sterilized with NaOCl (0.525%), plated on PDA, and incubated at 28 C. Percent germination was determined after 1 wk.

Toxicity of NH_3 to *P. omnivorum* sclerotia in soil. NH_3 was injected into soil-culture flasks after the air-dried soil (Houston black clay, pH 7.8) was mixed with a spatula to allow better distribution of the gas. NH_3 concentrations were 34, 69, 138, and 246 μg/g of soil. Two controls were included in this experiment: flasks in which the soil was mixed, but no NH_3 was added; and flasks in which the soil was undisturbed. After treatment, the flasks were incubated for 2 days, the sclerotia were washed from the soil, and the percent germination was determined. Three replications were run for each NH_3 concentration and control, and 150 sclerotia were plated from each flask to ascertain viability.

Toxicity of NH_3 to mycelium of *P. omnivorum*. Toxicity tests were conducted with mycelial mats from liquid cultures. Plugs (1 cm in diameter) were cut from mats and placed in Erlenmeyer flasks (125 ml), which were then sealed. NH_3 was injected into these flasks with a gas syringe to give final concentrations of 2, 4, 8, 12, or 20 μg/ml. Mycelial mats were exposed to NH_3 for 1 min. After exposure, each plug was removed from its incubation flask, washed in sterile distilled water, placed in a flask containing 25 ml of carrot juice medium (pH 5.69), and incubated at 28 C for 15 days. There were five replications of each treatment, and two controls. Neither control received any NH_3. One set of controls was dried at 100 C for 8 hr as soon as they were cut from the mycelial mat to determine original dry weight. The other set of controls was placed in growth medium as described above. Results are given as mean dry weight after 15 days of growth.

Toxicity of NH_3 to *P. omnivorum* in infected cotton roots. Infected roots were gathered from an area in the field where there were symptoms of root rot and prepared for NH_3 treatment the same day. All roots had numerous mycelial strands on their surface. Roots were cut into segments (2.5 cm long) and these were classified into three groups with diameters of 1.5, 1.0, and 0.6 cm, respectively. Roots were fumigated with NH_3 as described for mycelium. All roots were exposed to an NH_3 concentration of 56 μg/ml, and were incubated with the NH_3 for 1, 12, or 24 hr. Each

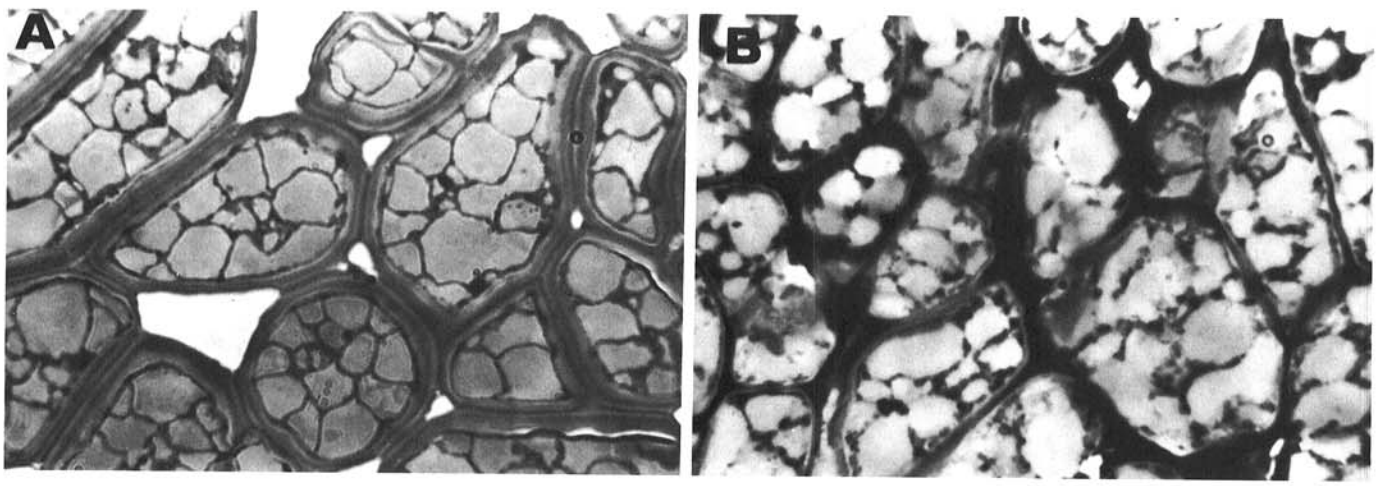

Fig. 1. Light microscopic cross sections of *Phymatotrichum omnivorum* sclerotia (×1,000). **A,** Untreated sclerotium with uniform staining of cell walls and membranes. **B,** Sclerotia treated with 56 μg NH_3/ml for 12 hr.

treatment was replicated five times with three root segments per replication. Following incubation with NH_3, the periderm was peeled from roots and the woody portion of the root was plated on PDA. Roots were examined for mycelial growth of *P. omnivorum* after 48 hr. Controls were treated exactly the same except the NH_3 was omitted. To determine the effect of ammonia on mycelial morphology, cross sections from treated and untreated roots were observed without fixation by means of a scanning electron microscope (JEOL JSM-U3).

Respiration of NH_3-treated mycelium. Plugs were cut from mycelial mats, exposed to NH_3 (4, 6, 8, 12, 20, and 40 μg/ml) for 1 min, and rinsed in sterile distilled water for 30 min. The average dry weight of these plugs was 10 mg. After the plugs were rinsed, the respiration rates of three of them were measured in a Warburg respiration flask containing 3 ml of carrot-juice medium, 0.2 ml of 0.1 N KOH in the centerwell, and 0.2 ml of 0.1 N HCl in a sidearm and maintained at 28 C. Treatments were applied in quadruplicate. Manometer readings were recorded hourly, and results are expressed as mean number of microliters of O_2 uptake per 10 mg (dry weight) of mycelial plugs.

Conductivity measurements of NH_3-treated mycelial plugs. Mycelial plugs were treated with NH_3 as previously described. Rates of NH_3 were 2, 4, 8, 12, 16, 20, and 40 μg/ml. Three plugs for each treatment were placed in 12 ml of deionized water, and conductivity readings were made every 15 min for the first 2 hr, and then every hour, with a YSI Model 31 conductivity bridge mated with a YSI #3402 conductivity cell (cell constant [K] = 0.1) (Yellow Springs Instruments, Yellow Springs, OH 45387). Three types of controls were included in these tests: mycelial plugs without NH_3 treatments; boiled plugs (5 min) without NH_3; and boiled plugs with NH_3 treatments. Each treatment was replicated three times. Average wet weight was determined for the mycelial plugs before NH_3 treatment, and conductivity is reported as absolute micro-ohms per milliliter.

RESULTS

Effects of NH_3 fumigation on sclerotia in vitro. All sclerotia exposed to 21 μg NH_3/ml or less had 100% germination (Table 1). At 28 μg NH_3/ml, the amount of sclerotial germination was a function of exposure time. Germination was only 33% at 56 μg/ml after 1 hr of exposure. NH_3 concentrations above 56 μg/ml, or longer exposure periods, completely inhibited sclerotial germination. Decreased germination was due to increased death of sclerotial cells. Treated sclerotia required longer germination time than untreated sclerotia, fewer hyphae were produced, and a dark-brown liquid was exuded. The amount of exudate increased in proportion to NH_3 concentration.

Cross sections of treated and untreated sclerotia were observed with a light microscope. Untreated, air-dried sclerotia had smooth, evenly stained cell walls, plasmalemma, and tonoplasts. Treated sclerotia (56 μg NH_3/ml) had intracellular disruption. Although the cell walls did not appear damaged or broken, they no longer stained as evenly as those of untreated sclerotia. The plasmalemma

TABLE 2. Germination of *Phymatotrichum omnivorum* sclerotia after fumigation with NH_3 in soil

NH_3 concentration (kg/ha)	NH_3 concentration (μg/g soil)	Mean germination[y]	Germination[z] (%)
0	0	14.9	99.7 a
28	34	14.1	94.0 b
56	69	12.7	85.0 c
112	138	9.8	65.0 d
224	276	3.4	21.0 e

[y] Mean germination from 10 plates (15 sclerotia per plate).
[z] Values followed by the same letter are not significantly different according to Duncan's multiple range test, $P = 0.05$.

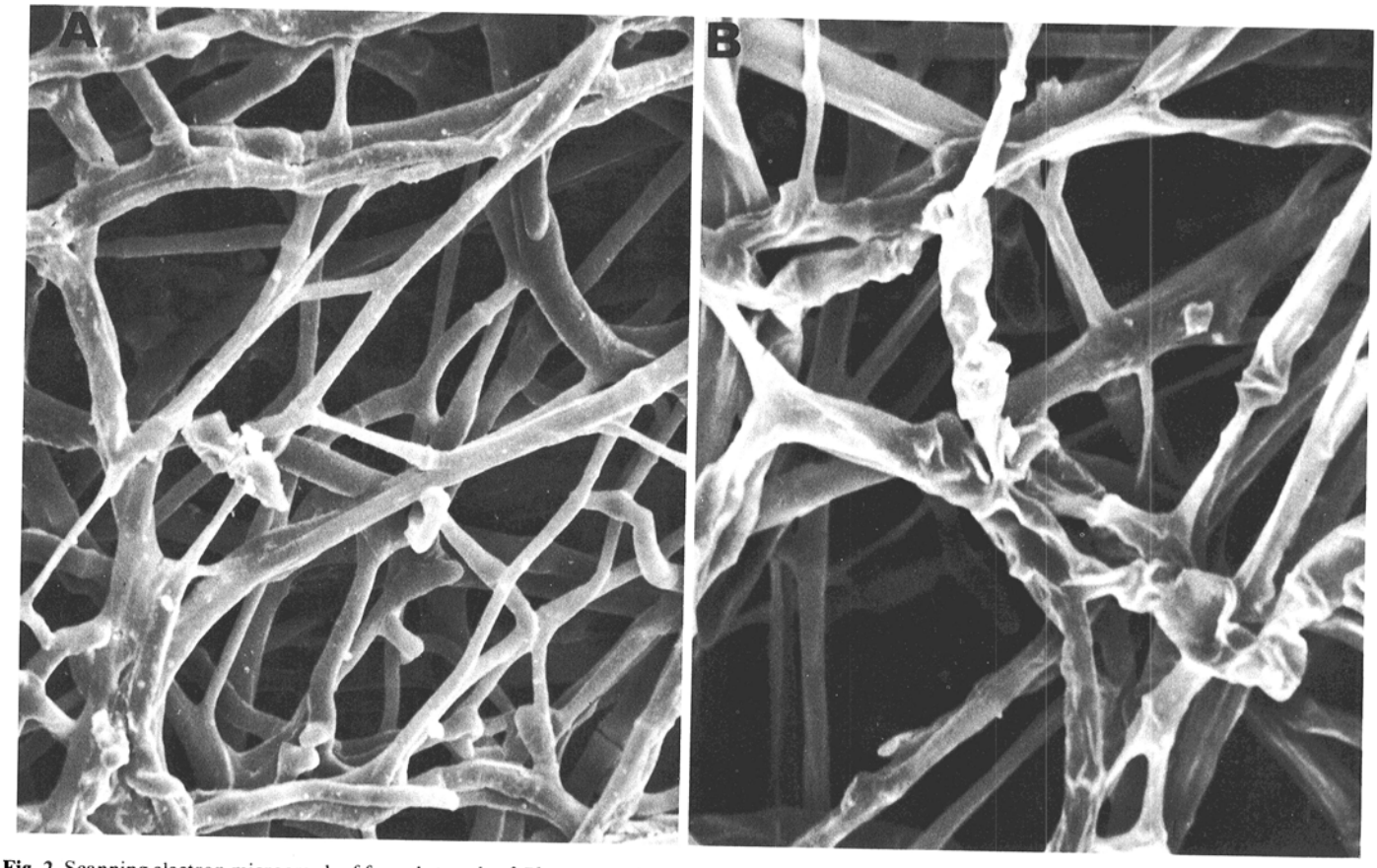

Fig. 2. Scanning electron micrograph of fungal strands of *Phymatotrichum omnivorum* on cotton roots before and after treatment with NH_3 (×2,000). **A,** Untreated hyphae. **B,** Hyphae exposed to 56 μg NH_3/ml for 12 hr.

and tonoplasts also appeared to be broken and distorted (Fig. 1).

Effects of NH_3 fumigation on sclerotia in soil. Comparable inhibition of sclerotial germination in soil required higher concentrations of NH_3 than did sclerotia in vitro (Table 2). With an ammonia concentration of 69 μg/g, germination was reduced to 85%. When treated without soil, exposure to 56 μg NH_3/ml of air reduced sclerotial germination by 67%. Concentrations of 69 or 138 μg NH_3/g of soil inhibited sclerotial germination by only 15 and 35%, but the resulting hyphae were sparse compared to those produced by untreated sclerotia.

Effect of NH_3 fumigation on mycelial plugs. Growth from mycelial plugs of *P. omnivorum* was severely inhibited by NH_3 fumigation. Exposure of mycelium to concentrations of NH_3 as low as 4 μg/ml resulted in further mass increase not significantly different from that of the heat-killed controls.

Mycelial growth of *P. omnivorum* from NH_3-treated roots. Roots that were incubated with NH_3 for 12–24 hr became dark brown to black. Mycelial growth from darkened tissue was sparse or absent. Scanning electron micrographs revealed that hyphae on treated roots had collapsed and showed considerably more breakage than untreated roots (Fig. 2).

Growth of *P. omnivorum* from untreated roots was affected more by duration of incubation with NH_3 than by root size (Table 3). Thickness of the root periderm was important in the 1-hr exposure. Periderm thickness was similar in the 1.5- and 1.0-cm-diameter roots, but was considerably thinner in the 0.6-cm-diameter roots, allowing faster penetration of the NH_3. There was no mycelial growth from roots after incubation with ammonia for 24 hr.

Effects of NH_3 on mycelial respiration. NH_3 inhibited mycelial respiration by 55% at 4 μg/ml, and 82% at 8 μg/ml (Fig. 3). Respiration with 8, 20, or 40 μg NH_3/ml were not significantly different, and were inhibited 82–86% compared to the control.

Effects of NH_3 on conductivity of mycelial plugs. The rate of electrolyte leakage, as determined by conductivity measurements, was highest during the first 15 min after NH_3 treatment (Fig. 4). Within the first 15 min, conductivity readings from all treatments, except the 16 μg/ml rate, were at least 40% of the maximum conductivity reached after 12 hr. Within 1 hr, all treatments except the 20 μg/ml rate were at least 70% of maximum. Maximum conductivity for all treatments occurred within 4 hr after exposure to NH_3. When plotted against NH_3 concentration, conductivity was not linear; this is indicative of massive membrane disruption. Final conductivity readings were all lower than the maximum readings.

TABLE 3. Mycelial growth of *Phymatotrichum omnivorum* isolated from naturally infected cotton roots of three diameters after fumigation with anhydrous ammonia (56 μg/ml)

Fumigation time (hr)	Mycelial growth of isolates from roots with diameters (cm) of:		
	1.5	1	0.6
Control	100[a]	100	100
1	60	66	13
12	26	26	13
24	0	0	0

[a] Each value is the mean percentage of roots showing growth 48 hr after incubation with NH_3. Means are averages of 15 replications.

DISCUSSION

The results of this study confirm the reported toxicity of NH_3 to *P. omnivorum* (15,16). It was interesting that large increases in NH_3 concentrations were necessary to kill air-dried sclerotia and mycelium. The fact that mycelium is more susceptible than sclerotia to low concentrations of NH_3 could have a direct effect on how and when field fumigations should be made. Early in this study, the proposed method for root rot control was to reduce sclerotial populations by applications of NH_3 in the spring just before planting. However, after toxicity studies were conducted on mycelium a new strategy was developed. The extremely low concentrations of NH_3, and short exposure periods required to kill the mycelium suggested that mycelium, and not sclerotia, should be the target of NH_3 fumigations. By applying NH_3 in the fall soon after cotton harvest, much of the mycelium, which would eventually form sclerotia, would be killed. This in turn would

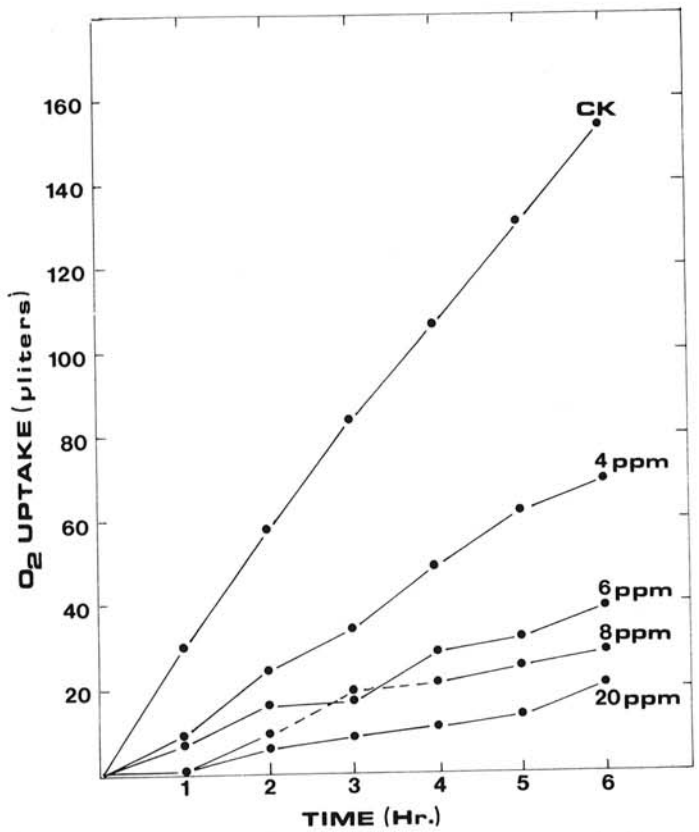

Fig. 3. Effects of various NH_3 concentrations on mycelial respiration of *Phymatotrichum omnivorum* measured as μl O_2 uptake per 10 mg (dry weight) of mycelium.

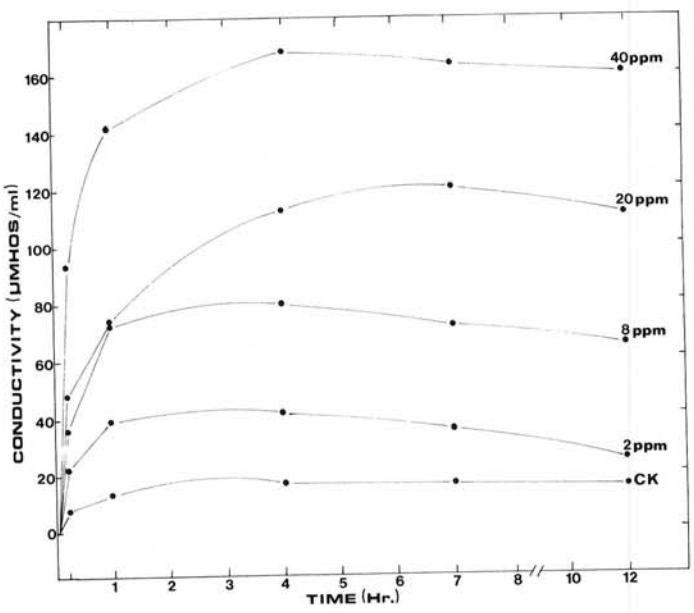

Fig. 4. Effect of NH_3 on electrolyte leakage from *Phymatotrichum omnivorum* mycelium over time. Approximately 30 mg (wet weight) of mycelium was used for each treatment.

reduce the inoculum density the following year, resulting in less disease and increased yields.

Most authorities agree that sclerotia are the primary structures involved in overseasoning (1,9,10), but hyphae surviving in crop residues have also been implicated (17,29). The data in Table 3 indicate that hyphae on cotton roots could be killed with NH_3 fumigation. Since *P. omnivorum* is a poor competitor, however, and crop residues are rapidly broken down in Blackland soils, fungus survival in crop residues has been of little concern.

Before any chemical compound is accepted for widespread use as a fungicide it is desirable to know its mode of action. Many studies have been conducted on the toxicity of various forms of ammonia to fungi, but few on the actual mechanism of toxicity. Two exceptions are the reports by Vines and Wedding (28) and Henis and Chet (6). Vines and Wedding (6) reported that ammonia toxicity was due to inhibition of the electron transport system, specifically the inhibition of NADH oxidation. Henis and Chet stated that NH_3 toxicity to *Sclerotium rolfsii* was due to nonoptimum soil pH resulting from NH_3 fumigation. However, neither of these can adequately explain some of our recent observations following treatment of sclerotia and mycelium of *P. omnivorum* with NH_3.

After treating sclerotia with NH_3, a dark brown exudate was observed and when NH_3 concentrations increased, electrolyte leakage also increased. Furthermore, this leakage began immediately after exposure to NH_3 with the maximum rate occurring within the first 15 min. This suggested a direct effect of NH_3 on cellular membrane systems. Observations of NH_3-treated sclerotia with the light microscope enforced this idea.

Vines and Wedding reported that NH_3 inhibited respiration by specifically inhibiting the oxidation of NADH. We also found that after exposure to NH_3, respiration was reduced. However, in view of the conductivity studies, we feel that toxicity of NH_3 to *P. omnivorum* at the concentrations used, is the result of a more physically disruptive response than inhibition of NADH oxidation. We also believe that NH_3 exerts its toxic effect directly on the fungus and not indirectly by altering the pH of the substrate. These ideas are enforced by the results in Tables 1 and 2. It is our belief that NH_3 toxicity to *P. omnivorum* is due to direct disruption of the fungal membrane systems, and that the amount of disruption depends on the initial NH_3 concentration to which the fungus is exposed. A more detailed explanation of the toxic mode of action of NH_3 on fungi is needed. We are conducting further studies on the toxic effects of NH_3 on *P. omnivorum* membranes; results of some of these have already been reported (20,21).

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